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BLACK GALL ON ASPEN:
ANATOMY, HISTOLOGY, AND RELATIONSHIP TO DECAY

by

PATRICIA ELLEN CRANE, B.Sc.

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

AND RESEARCH IN PARTIAL FULFILLMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

IN FOREST PATHOLOGY

DEPARTMENT OF PLANT SCIENCE

EDMONTON, ALBERTA

FALL 1993

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled BLACK GALL ON ASPEN: ANATOMY, HISTOLOGY, AND RELATIONSHIP TO DECAY submitted by Patricia Ellen Crane in partial fulfillment of the requirements for the degree of Master of Science in Forest Pathology.

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Date: October 4, 1993

DEDICATION

To my parents, Violet and Ted Thomas

ABSTRACT

The annual harvest of trembling aspen (Populus tremuloides) in Alberta has increased dramatically over the past 12 years, but its use is often limited by the presence of extensive decay and stain. Aspen with black stem galls of unknown cause reportedly have less advanced decay than non-gall trees. Nine field sites having high numbers of galled aspen were surveyed for presence of Phellinus tremulae conks. Logistic regression showed that the odds of a gall tree having at least one conk were only 44% of those for trees without galls, and that likelihood of conks varied among sites and increased with increasing tree diameter. A morphological study of 14 stem galls, including several thought to be caused by the poplar budgall mite (Aceria parapopuli), showed that mite galls differed in surface and internal characteristics from galls in the study plots. A detailed anatomical and histochemical study was done of small branch galls, normal and gall wood and bark. Light microscopy showed that the cambium of black galls produces greater numbers of cells per growth ring and that vessel elements and fibers are unusually small and misshapen. Gall xylem had characteristics associated with wounding or infection: phenolic deposits in ray cells, tyloses and fibrous material in vessel elements. Frequent radial strands of undifferentiated callus tissue surrounded by necrophylactic periderms indicated sites of annual cambial damage of unknown cause. White areas within dark gall xylem were free of most of these abnormalities, suggesting reversion to normal cambial divisions and that a persistent agent is required for continuing tumor growth. Thickened outer bark always harbored a variety of saprophytic fungi, especially hyphomycetes. The absence of obvious bacteria or fungi within living tissue suggested

they are not involved in tumorigenesis. It is probable that different black stem galls have different causes, such as insects or mites, viruses, MLO's, or Agrobacterium sp.

ACKNOWLEDGEMENTS

I am grateful to my supervisors, Y. Hiratsuka and P. Blenis, for their wisdom and thoughtful guidance during my studies, and for allowing me the freedom to work at my own pace. I thank D. Cass and R. Currah, members of my graduate committee, for their excellent advice during my project.

It was a privilege to conduct my research in the excellent laboratory facilities of the Northern Forestry Centre. My studies were made especially pleasurable by the friendship of co-workers there. I thank P. Maruyama for sharing his expertise in the field and laboratory; L. Hutchison for sharing his mycological knowledge; K. Jacubec for field and computer assistance; and P. Chakravarty, G. Herger, and K. Mallett for their encouragement. I am grateful for financial support from Forestry Canada under the BIOCOND Network and from NSERC Strategic grant ATR0101490. The regular meetings of the multidisciplinary group brought together by this grant, including W. Ayer and colleagues, Department of Chemistry, University of Alberta; and L. Sigler, University of Alberta Microfungus Collection, provided an excellent forum for presentation of my research results.

My return to academe was made possible by the good will of my family. I am deeply grateful to my husband, Adrian, for his love and support, and to my children, Stephanie and Jonathan, whose good humor and faith kept me going.

TABLE OF CONTENTS

Chapter	Page
I. INTRODUCTION	1
General characteristics and distribution of aspen	1
A commercially important species	3
Limitations on usage of aspen	4
Black galls and the potential for disease control	9
Hypotheses and objectives	11
II. RELATIONSHIP OF BLACK GALLS TO DECAY BY PHELLINUS TREMULAE	12
Introduction	12
Methods and materials	12
Results and discussion	16
III. ANATOMICAL AND HISTOCHEMICAL COMPARISON OF NORMAL AND GALL WOOD AND BARK	22
Introduction	22
Methods and materials	23
Gross morphology and comparison with known poplar galls and stem swellings	23
Light microscopy	25
Fixation, dehydration, and paraffin embedding	25
Histochemistry	27
Phenolics	27
Lignin and suberin	27
Calcium oxalate	28
Width of growth rings and cell shape and size	28
Examination for microorganisms, insects, and mites	29
Scanning electron microscopy	30

Chapter	Page
Results	30
Macroscopic features of black galls	30
Microscopic comparison of normal stem tissue and gall tissue	35
Outer bark	35
Phloem	39
Cambium	43
Xylem	47
White islands	56
Wood callus and possible infection points	56
An "atypical" gall	60
Search for young galls	60
Insect galls	60
Diplodia galls	61
Branch and twig swellings on gall trees	61
Presence of microorganisms	65
Discussion	69
Summary of major anatomical features of black galls and comparison with other galls	69
Xylem	69
Cambium	71
Phloem	72
Wood callus and dead cambium	73
Resistance reactions	74
Thickened bark, necrophylactic periderms, and suberization	75
Lignin and phenolics	78
Tyloses and vascular occlusion	80
Calcium oxalate crystals	82
Nature of causal agent	84
Fungi and bacteria	84
Insects and mites	87
Viruses and mycoplasma-like organisms	89
Clues from branch galls	90
IV. GENERAL DISCUSSION AND CONCLUSIONS	92
Nature of tumorigenesis in black galls	92
Black gall and aspen decay	94
LITERATURE CITED	96
APPENDIX: Glossary of anatomical terms	108

LIST OF TABLES

Table		Page
1.	Gross morphology of 14 black galls	24
2.	Comparison of quantities of phenolic compounds in normal and gall tissue	49
3.	Comparison of cell size, ray height, and width of annual growth rings from normal and gall xylem	50

LIST OF FIGURES

Figure **Page**

1. Current natural distribution of aspen in North America 2
2. (A) Advanced decay caused by Phellinus tremulae in aspen. (B) Fruiting body of P. tremulae. (C) Black stem galls on mature aspen 8
3. Locations of black galls found during the field survey 14
4. Percentage in each plot of gall and non-gall trees with P. tremulae conks 18
5. Percentage of trees in each plot with P. tremulae conks, according to diameter class 19
6. (A) Gall caused by Aceria parapopuli. (B) Black stem gall. (C) Cross section of mite gall. (D) Cross section of black gall. (E) Sap exudate, gall cross section. (F) Dead cambium beneath gall bark 34
7. Bark. (A, B) Normal. (C-F) Gall 38
8. Phloem. (A) Normal. (B) Fibers. (C, D) Sclereids. (E, F) Phloem initials 42
9. Cambium. (A, B) Normal. (C, D) Gall. (E, F) Xylem initials 46
10. Xylem. (A) Normal radial section. (B) Gall radial section. (C) Whorled cells in gall 53

Figure **Page**

11. Xylem. (A, B) Tyloses in gall vessels. (C, D) Macerated cells, normal and gall. (E) Phenolics in gall. (F) White island in gall. (G) Calcium oxalate crystals, gall 55

12. Cambial damage. (A) Callus near cambium. (B) Suberized cork. (C) Rapidly dividing cells. (D) Plasmolyzed cells. (E) Phenolics in necrotic area. (F) Wood callus. (G) Lignified cells in wood callus. (H) Fluorescing cork cells in periderm around wood callus 59

13. Small branch galls. (A) Various sizes. (B) Brown region inside gall. (C) Dead center of brown region surrounded by periderm. (D) Lateral necrotic region. (E) Degraded branch center below gall 64

14. (A) Hyphomycetes on gall surface. (B) Fruiting body, possibly Melanomma sp., on surface of branch gall. (C) Vessel pit border with mottling, large stem gall 68

LIST OF ABBREVIATIONS

BR	Blue Ridge
b	border cells
br	branch stub
C	cambium
ca	callus
CTMP	chemithermomechanical pulp
d	dying cells
dr	druse crystals
DNA	deoxyribonucleic acid
DV	Drayton Valley
ex	sap exudate
FAA	formalin - acetic acid - ethanol
fi	fusiform initials
g	granular material
HV	Hoepfner-Vorsatz method
IAA	indoleacetic acid
lf	libriform fiber
LV	Lakeview Trail, Elk Island National Park
MLO	mycoplasma-like organism
nec	necrotic region
np	necrophylactic periderm
n	nucleus

OSB	oriented strand board
P	phloem
pf	phloem fibers
ph	phenolic compounds
pl	starch plastids
PO	peroxidase
PPO	polyphenoloxidase
pr	prismatic crystals
r	ray or ray cell
rh	rhytidome
ri	ray initials
RLS	radial longitudinal section
SFG	safranin - fast green
sc	sclereid
si	sieve plate
t	tylosis
TLS	tangential longitudinal section
TS	transverse section
v	vessel
w	white band or island
X	xylem

CHAPTER I. INTRODUCTION

General characteristics and distribution of aspen

Populus tremuloides Michx. has been known by many names: abele, aspen, white, or smooth-barked poplar; quaking, trembling, golden, mountain, or Vancouver aspen; or simply as popple or asp (Peterson and Peterson 1992; Strothmann and Zasada 1957). A member of the willow family (Salicaceae), it is the most widely distributed tree species in North America. Its transcontinental range covers 110 degrees of longitude and 40 degrees of latitude (Strothmann and Zasada 1957) (Fig. 1), over which its external appearance varies markedly. Although it occurs throughout Alberta, it attains its best development in the mixedwood forests of the Boreal Forest Region in central and northern parts of the province (Thomas et al. 1960). Aspen makes up 85% of Alberta's growing stock of hardwood species. Other parts of its commercial range include central and northern Saskatchewan and Manitoba, the Lake States, and parts of New England.

Trembling aspen occurs as a dominant or codominant tree on a wide range of sites, but is mainly a species of well-drained uplands, achieving its best growth on moist, well-drained, porous, loamy soils (Peterson and Peterson 1992; Strothmann and Zasada 1957). As a fast-growing, shade-intolerant species, it often grows in pure stands when young, but is usually succeeded by more tolerant conifers and hardwoods (Hosie 1969; Jones and Shier 1985). Common associates in Alberta are balsam poplar (Populus balsamifera L.), white birch (Betula papyrifera Marsh.), and white spruce (Picea glauca (Moench) Voss). Stands of clonal origin predominate, and clones vary in morphology, tree quality, physiological responses, and decay levels (Hiratsuka and Loman 1984;

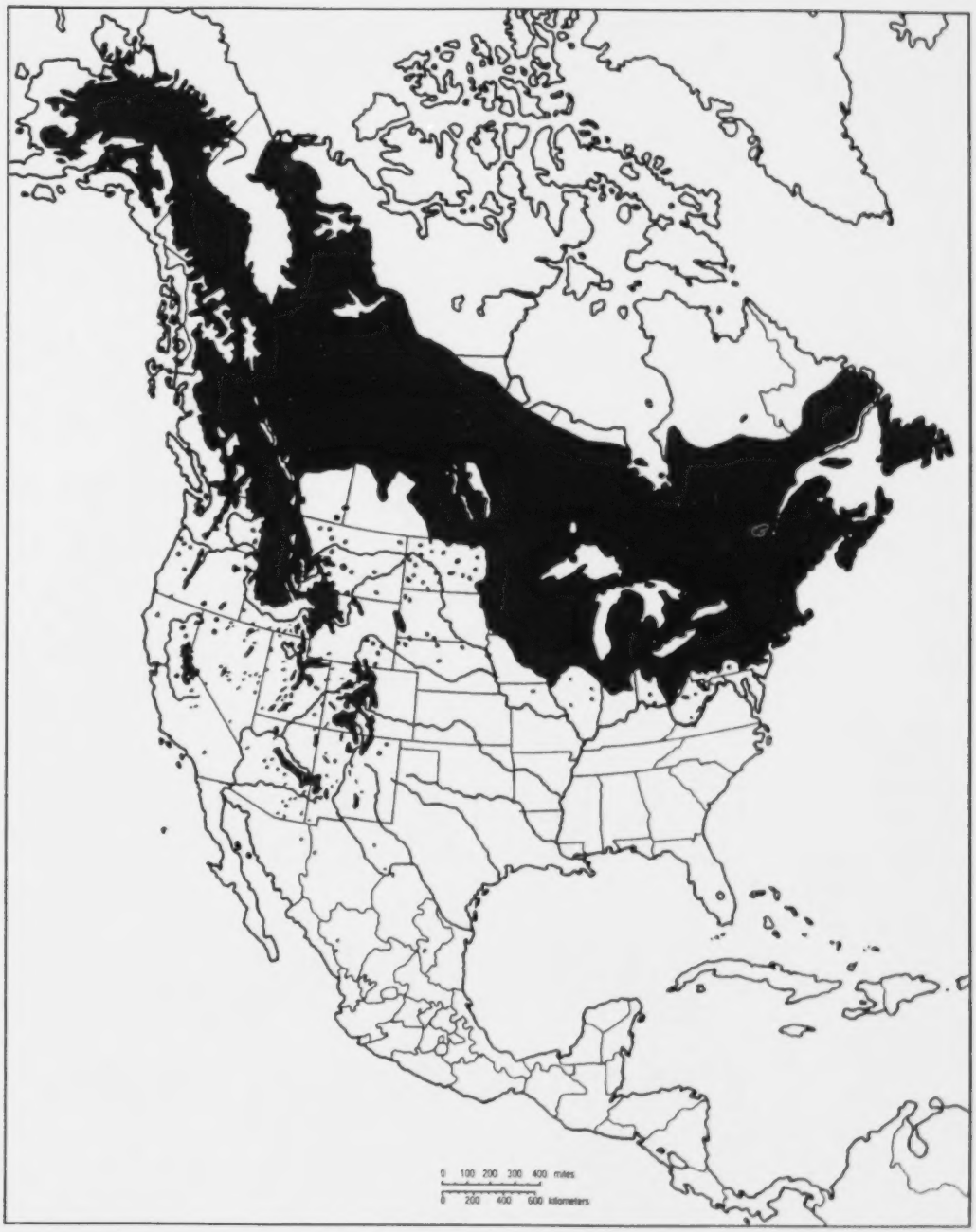


Fig. 1. Current natural distribution of aspen in North America (from Peterson and Peterson 1992).

Peterson and Peterson 1992). The roots are long-lived, and survive by regeneration of inconspicuous suckers even in predominantly conifer stands.

A commercially important species

A remarkable shift in attitude about the commercial usefulness of poplar (mainly aspen) has occurred in the past decade. It was once regarded as a weed species that hindered efficient harvesting of the more valuable softwoods. In Alberta, poplar accounted for only 2.4% of the total timber harvest in 1980; however, this had increased to 18% by 1992 (Alberta Energy and Natural Resources 1982-86; Alberta Forestry, Lands and Wildlife 1989-92). Some of the reasons for this change are an increasing scarcity of economically accessible softwood timber in Canada; recognition of some of the unique anatomical, chemical, physical, and mechanical properties of aspen; and development of new technologies to use this resource more efficiently.

The development of oriented strand board (OSB) using aspen in the early 1980's has been a success story in Alberta. In the manufacture of OSB, large, thin strands of aspen are bonded together under intense heat and pressure. Aspen's low density is an advantage in this process, since moderate pressure brings particles into close contact, ensuring a board with good strength (Ondro 1989; Peterson and Peterson 1992).

Aspen has recently become prominent in the pulp industry of the prairie provinces and northeastern British Columbia and is now considered an excellent raw material for pulp and paper. Its appeal in these industries is a result of its low lignin and high carbohydrate content. Low lignin content permits wood to be softened and delignified

more easily than wood of conifers. It is used to make both chemical (kraft) pulps and mechanical, especially chemithermomechanical (CTMP), pulps. Some of the new large CTMP pulp mills claim to be chlorine-free, zero-effluent mills using aspen as raw material. The slender, short aspen fibres produce a pulp that is ideal for fine paper grades that are smooth and opaque. Advances in technologies for processes such as peroxide bleaching have resulted in a significantly better quality product made in mills with low capital costs and with half the fiber requirements of older kraft mills (McCready 1992; Stevenson 1991; Cheyne 1990).

Secondary forest products for which aspen is being used are pallets, cartons, corrugated boxes, firewood, and cattle pellets. Its naturally white, splinterless wood means that it may have excellent potential in the furniture industry, while its lightweight, tough, tasteless, and odorless qualities make it ideal for chopsticks (Ondro 1989; Peterson and Peterson 1992).

Apart from these commercial uses, aspen is valued for its aesthetic qualities such as fall coloration, its fast growth, self-pruning habit, and easy care in parks and urban settings. This species and its well-developed understories of shrubs, forbs, and grasses provide important habitat and food source for native ungulates such as moose, white-tailed deer, and elk, and smaller mammals, birds, and insects (Peterson and Peterson 1992).

Limitations on usage of aspen

The most severe limitation on economic utilization of aspen is its lack of resistance to wood decay and staining organisms. Heartwood decay by fungi is the most

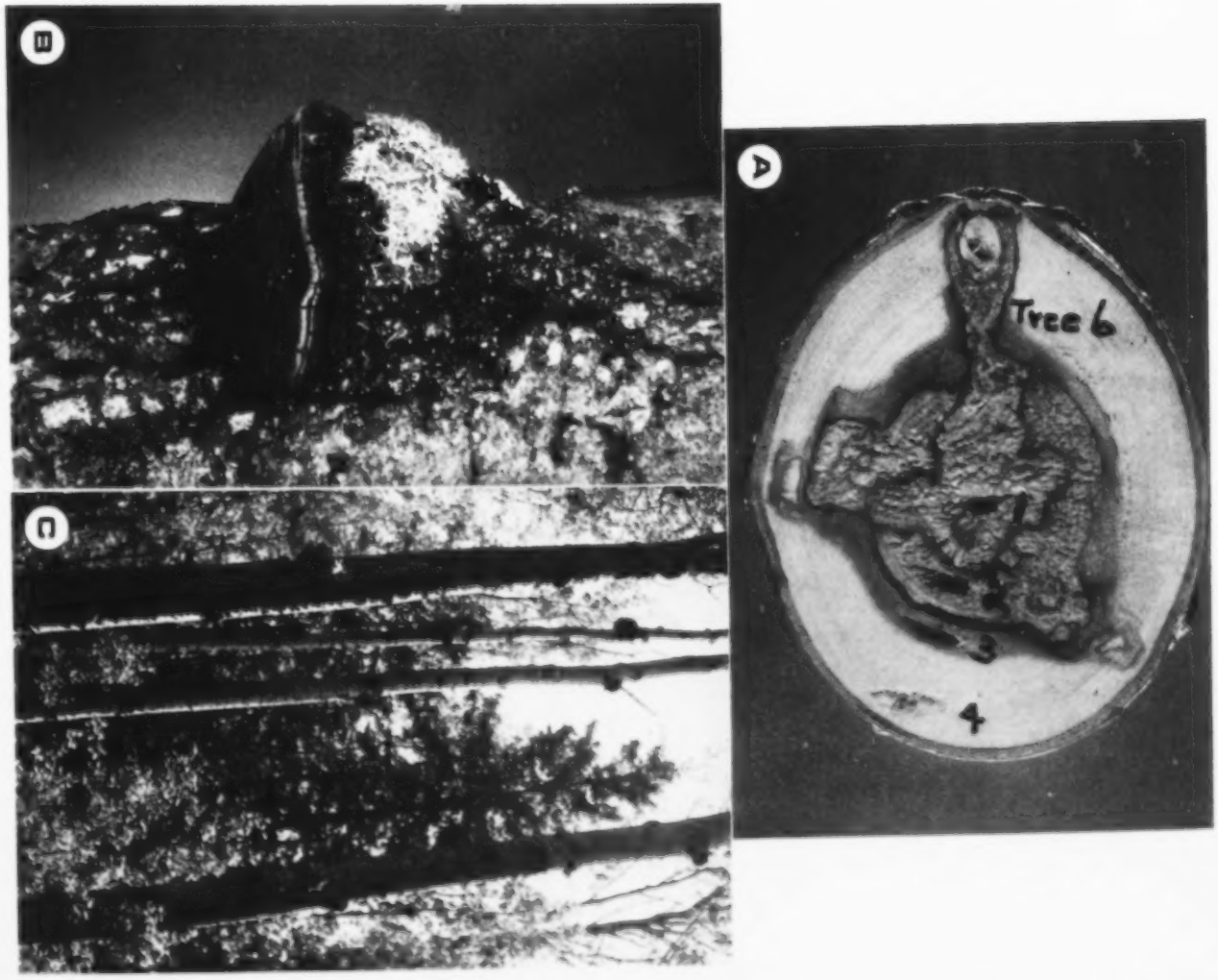
important cause of fiber loss in living poplars (Kennedy 1968; Cheyne 1990), and is an important consideration in determining the age at which a stand should be harvested. Decay tends to increase with age of trees, and given that 80% of Alberta's aspen forests are mature to overmature (over 80 years old), a substantial portion of aspen forests are affected (Hiratsuka and Loman 1984; Ondro 1989; Thomas et al. 1960). More than 250 species of fungi cause or are associated with decay in North American aspen, but most of these are of minor importance in living trees (Lindsey and Gilbertson 1978). Of the 27 species identified by Thomas et al. (1960) as causing decay of live aspen in Alberta, Phellinus tremulae (Bond.) Bond. & Boriss. (= Fomes igniarius (L.:Fr.) Kickx) is by far the most important. In their survey of 835 living aspen in mixedwood forests of the Boreal Forest Region, 73% were decayed, with about one-third of the rot caused by this fungus. Similar results have been reported by other authors (e.g. Basham (1960) in Ontario and Hinds and Wengert (1977) in Colorado). P. tremulae, known as the false tinder fungus, causes a white trunk rot, and decay columns are surrounded by characteristic brown to black lines (Fig. 2A). The main indicators of its presence are the perennial, hoof-shaped conks with cracked, blackish upper surfaces, often located at branch scars on the tree stem (Fig. 2B). This pathogen is thought to gain entry to the tree through trunk wounds such as rotten branch stubs and stem cracks caused by frost (Hiratsuka and Loman 1984; Hiratsuka et al. 1990). Other important disease or decay organisms of aspen in Alberta are Peniophora polygonia (Pers.:Fr.) Boud., found in decayed or discolored wood; Armillaria ostoyae (Romag.) Herink, Ganoderma applanatum (Pers. ex Wallr.) Pat., Fomitopsis pinicola (Sw.:Fr.) P.Karst., and Gymnopilus spectabilis

(Fr.:Fr.) A.H. Smith, causing root and butt rot; and yeasts, bacteria, imperfect fungi, and ascomycetous fungi such as species of Ceratocystis and Verticicladiella, causing staining in standing trees or stored wood (Hiratsuka et al. 1990).

The amount of decay and stain that can be tolerated varies with the end-use. Although the manufacture of OSB will tolerate stained wood, excessively decayed wood does not cut cleanly, thickness control is poor, more drying and more resin are required, and bending strength of boards is lowered (Peterson and Peterson 1992). Kraft pulping is fairly tolerant of decayed wood, but since severely rotted wood is often lost during the chipping process, costs are increased when material that is brought to the mill is subsequently discarded. Wood quality is more important in mechanical pulping, such as CTMP, since stain and incipient decay reduce fiber yields and require the use of more bleaching chemical, adding greatly to the costs (Cheyne 1990; Peterson and Peterson 1992).

Decayed wood is least tolerated in solid wood products. Several other characteristics of aspen may also restrict its use in solid wood products, including its poor dimensional stability during drying, presence of wetwood (usually associated with bacteria), uneven drying, low yields because of small log size, and enormous quantities of residue (Peterson and Peterson 1992). The most efficient utilization of aspen occurs when several complementary industries or subsidiary markets can be situated close together to make use of all the fiber available.

Fig. 2. (A) Advanced stem decay caused by Phellinus tremulae. (B) A fruiting body of the decay fungus P. tremulae on an aspen stem. (C) Black stem galls on mature aspen near Blue Ridge, Alberta.


Tree 6
3
4

Black galls and the potential for disease control

Problems of decay and stain are compounded by the inability to predict their presence in standing trees. Apart from conks of P. tremulae, indicators used to predict presence of decay are mushrooms of A. ostoyae, fruiting bodies of P. polygonia, basal tree damage, and rotten branch stubs. These, however, do not provide a reliable estimate of defect volume (Maier and Darrah 1989; Hiratsuka et al. 1990).

A greater understanding of the interactions of decay and stain organisms and the circumstances under which they invade aspen is required. This may allow development of strategies to control their negative effects on this important forest resource.

Rare stem deformities known as black galls offer a unique opportunity to study decay-resistance mechanisms in aspen. These tumorous growths occur on aspen stems in many places in Alberta, but usually in isolated groups (Fig. 2C). Few references to these structures have been found in the literature, and they do not seem to have been studied extensively, having been considered of minor importance except where tree appearance is important (Ostry et al. 1989). They appear to be fairly common in Colorado: Juzwik et al. (1978) found that in 30 sites surveyed, 27% had aspen trees with burls or perennial trunk galls of unknown cause. Hinds (1985) showed photographs of globose trunk galls, which he assumed to be insect related, and rough "clinker-like" trunk galls. Ostry et al. (1989) indicated that stain and decay may be associated with them. Peterson and Peterson (1992) claimed that these deformities consist of raised callus tissue that developed where superficial wounds have occurred. However, Hiratsuka and Loman

(1984) suggest that wood of black galls is sound and is seldom associated with advanced decay caused by P. tremulae. Near Whitecourt, Alberta, they found unsound wood in only 1 of 20 large aspen trees with black galls, a much lower proportion than would be expected (Thomas et al. 1960). This observation has kindled interest in understanding the nature of black stem galls. If trees with black galls do, indeed, have a lower decay incidence than surrounding non-gall trees there are several possible reasons: (1) the causal organism of black galls produces metabolites that protect the tree from infection by decay organisms or from disease development; (2) compounds produced by the tree in response to organisms present in the black galls enhance decay resistance; (3) trees having black galls are genetically resistant to decay; or (4) presence of decay reduces the likelihood of gall formation.

Substantial progress has already been made in investigating the potential for environmentally acceptable biological control measures for stain and decay in aspen. Isolations from black galls and from healthy, stained, and decayed aspen wood over 3 years have yielded over 100 species of fungi, yeasts, and bacteria (Hutchison and Hiratsuka 1992, and personal communication). Included are several new species of fungi that are exclusive to black galls and related canker-like structures: the yeast-like Hyphozyma lignicola (Hutchison et al. 1993), Phoma etheridgei (L. Hutchison, personal communication), and the black-yeast-like hyphomycete Capnohughesia nigra (L. Hutchison, personal communication). Among the isolated organisms, several potential biological control agents have been identified. Lecythophora hoffmannii (van Beyma) W. Gams & McGinnis and Stachybotrys cylindrospora C.N. Jenssen, both from healthy wood,

are antagonistic to Ophiostoma crassivaginatum (H.D. Griffin) T.C. Harrington (=Ceratocystiopsis crassivaginata (H.D. Griffin) Upad.), the most common cause of blue sapwood stain in aspen. These hyphomycetes appear to inhibit growth of the pathogen on wood chips by producing fungitoxic compounds, some of which have been identified (Chakravarty and Hiratsuka 1993; Hiratsuka et al. 1993; Ayer and Miao 1993). Similarly, several studies have confirmed that the growth of Phellinus tremulae is substantially reduced in culture and in aspen stems when the less destructive decay fungus Peniophora polygonia is present (Hiratsuka et al. 1990; Chakravarty and Hiratsuka 1992; Trifonov et al. 1992). Phoma etheridgei, on the other hand, is strongly antagonistic to both Phellinus tremulae and Ophiostoma spp. (L. Hutchison, personal communication). In addition to the above studies, biochemical analysis of black gall tissue is in progress.

The current study will complement these investigations. My objectives were (1) to determine whether trees having black galls are less likely to be decayed than surrounding trees without galls; and (2) to compare the morphology and anatomy of normal and gall bark, phloem, and wood and to interpret the unique features of these tissues in light of similar characteristics found in other pathological systems. This study may provide information relevant to an understanding of black gall etiology.

CHAPTER II. RELATIONSHIP OF BLACK GALLS TO DECAY BY PHELLINUS TREMULAE

Introduction

There is evidence that aspen trees with black galls have less decay than those without galls (Hiratsuka and Loman 1984). If this is true, a negative correlation between galls and decay might lead to better methods of predicting or reducing decay. New research approaches and opportunities for reducing decay losses might result if it could be shown that gall induction caused a decreased likelihood of decay development. This is especially significant, since currently there are no methods for reducing incidence of decay other than attempting to minimize wounding. Decays cause a large economic loss, and even a small reduction in decay frequency would represent a large economic gain. Even in the absence of a causal association between galls and decay, a strong statistical association might be valuable in improving methods for decay prediction. Given the importance of reliable estimates of merchantable volume, any methods for improving accuracy would be valuable.

A field survey was undertaken to test the hypothesis that trees with black galls are less likely to be decayed by Phellinus tremulae than are surrounding non-gall trees.

Methods and materials

Information on location of gall trees came from many sources, including forest industry spokesmen, ranger reports, and scientists doing related work. Nevertheless, it was difficult to find enough gall trees in one area to compare to surrounding non-gall

trees. The resulting distribution map (Fig. 3) shows locations of black galls used in the survey as well as other locations that were confirmed either by photograph or personal observation.

The second problem was how to assess presence or absence of advanced decay in a simple manner. At Blue Ridge, Alberta, Hiratsuka and Loman (1984) found that 94.5% of aspen with conks and/or large scars had a significant amount of advanced decay, but only 16.7% of trees without such external indicators had advanced decay. Similarly, Basham (1958) found that an average of 86.2% of trees with one or more P. tremulae conks had advanced decay. Since conks of P. tremulae are considered the most reliable indicator of internal decay that would lead to a reduction in usable volume (Basham 1958; Hiratsuka et al. 1990), decay was evaluated solely on their presence.

There was also some difficulty in defining a black gall, since the thinness of aspen bark makes it prone to injury and disease by many agents, causing rough bark and thickening. In addition, aspen's self-pruning habit results in many branch scars and stubs along mature stems, and in some clones these are accompanied by obvious swellings. The structures referred to as "black galls" were usually, but not always, globose, with a rough, black, fissured surface. Without a known cause, it was impossible to characterize them beyond this general definition. During the field study, a large area (>l ha) was found in northeastern British Columbia near Dawson Creek in which aspen were infected with large multiple stem galls having fresh red-colored bud proliferations on their surfaces. These buds contained poplar bud gall mites, Aceria parapopuli Keifer, which usually occur on twigs, rarely on stems (Campbell et al. 1969). It was concluded that

Fig. 3. Locations of black galls in Alberta and British Columbia identified during this study. ●Plot used in the field survey. ■Other confirmed location of stem galls, identity unknown. ○Stem galls caused by the mite Aceria parapopuli.

there are at least two different types of large stem tumors found on aspen in western Canada. A more detailed description of these types will be given in Chapter III. It is believed that all plots in this field study contained galls of the same type, not caused by A. parapopuli, since surface bud proliferations were not present.

Nine sites in central Alberta with a high concentration of black galls were surveyed: three at Elk Island National Park (Moss Lake and Lakeview (LV) 1 and 2), four near Blue Ridge (BR), one near Edson, and one near Drayton Valley (DV). A plot was established at each site to include all aspen trees with black galls and any interspersed or nearby trees without galls. The plots varied in size, and natural boundaries such as fences or swamps were often used to delimit them. P. tremuloides was the dominant tree on all sites, although most included some white spruce, balsam poplar, and occasionally willow and white birch.

The outside diameter of the stem, 1.3 m aboveground, was measured for each tree. Because of the difficulty of counting growth rings in aspen, especially when they are decayed, and because decay levels may be more accurately predicted by diameter than by age (Maier and Darrah 1989), no attempt was made to determine tree age. At least two observers agreed on the numbers of conks and galls per tree. To ensure more accurate observation of tree tops, binoculars were used.

Logistic regression (SAS Institute 1989; Hosmer and Lemeshow 1989) initially was used to model the likelihood of a tree having at least one conk as a function of the presence or absence of at least one gall, tree diameter, site, and all two- and three-way

interactions of these factors. Subsequently, non-significant effects were eliminated from the model to arrive at the most parsimonious model that adequately fit the data.

Results and Discussion

A total of 529 trees were assessed in the 9 plots. There were 1 to 15 galls per tree, with 36%, 47%, and 17% of the galls present in the bottom, middle, and top thirds of trees, respectively. Figure 4 summarizes the data for all plots, regardless of diameter.

The final model for predicting the probability of a tree having at least one conk was

$$C = -1.9774 - 0.8107\ \text{gall} + \sum y_i \cdot \text{site}_i + 0.4062\ \text{diameter},$$

where C = ln(odds of a tree having at least one conk),

gall = an indicator variable that equals 1 if a tree has a gall and 0 otherwise,

y_i = a coefficient for the site effect,

site_i = an indicator variable that equals 1 for the i^{th} site and 0 otherwise, with the ninth site set to 0 and used as the reference site,

diameter = stem diameter, in dm, 1.3 m aboveground.

Neither the three-way interactions nor any of the two-way interactions were significant, and using number of galls instead of the presence or absence of galls did not improve the model fit.

Trees with galls were less likely to have conks than trees without galls (Figs. 4, 5). The coefficient of -0.8107 for the gall effect indicates that the odds of a gall tree

having at least one conk were only $e^{-0.8107}$ or 0.44 (=44%) times as great as for a tree that was free of galls. The 95% confidence interval for this odds ratio was 28.6% to 69.1%. The absence of a significant site-by-gall effect indicates that the association between the presence of galls and the absence of conks was relatively consistent across sites, even though there were two sites in which conks were more common on gall than non-gall trees.

The likelihood of conk formation varied with site (Fig. 4). The Moss Lake site had the highest occurrence of trees with conks; the odds of a conk being present were 4.1 times as great as on the reference site. At the opposite extreme, the odds of a conk being present on a tree at the BR1 site were only 0.75 times as great as on the reference site. This location effect is likely related to the difference among aspen clones in decay susceptibility. It has been shown that there is a highly significant difference in aspen clones in percentage and volume of decay; this variation is more important than site, based on percentage decay in intermingled clones on the same site (Hiratsuka and Loman 1984; Wall 1971). Although it was not proven that all trees within each site were from the same clone, it is likely that this was the case for most of them because of similar bark characteristics. The concentration of black galls in isolated groupings also supports the notion of a clonal susceptibility to these defects.

The likelihood of trees having conks increased with stem diameter (Fig. 5), as would be expected, given the previously demonstrated positive association between diameter and stem decay (Maier and Darrah 1989). The regression coefficient of 0.4062 indicates that each 1 dm increase in stem diameter increases the odds ratio by a factor of

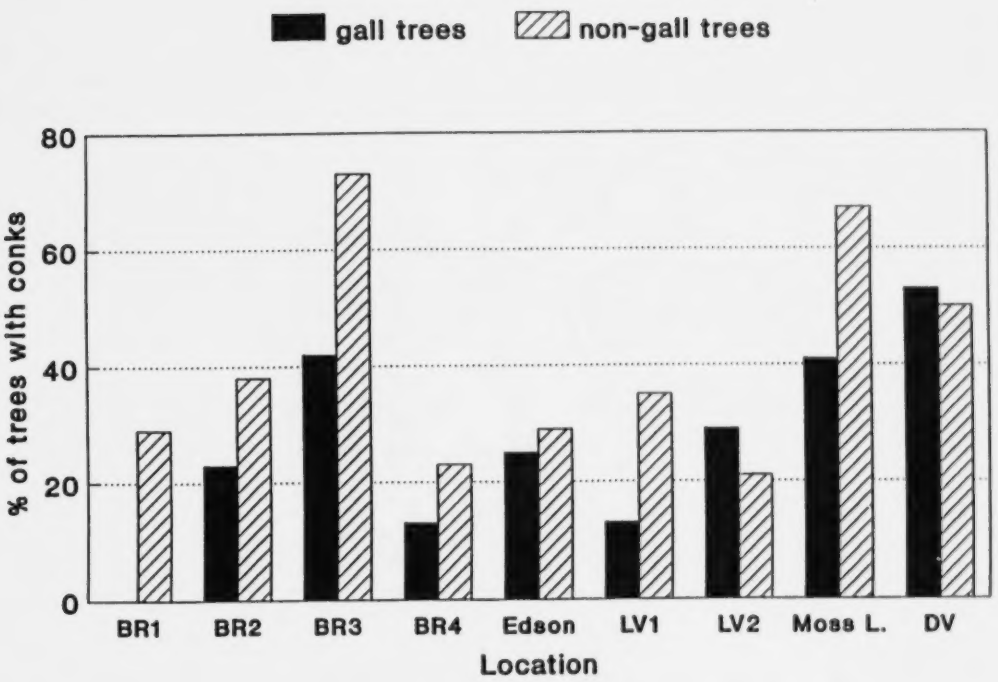


Fig. 4. Percentage in each plot of gall and non-gall trees with advanced decay, indicated by presence of P. tremulae conks. BR, Blue Ridge; LV, Lakeview Trail, Elk Island National Park; DV, Drayton Valley.

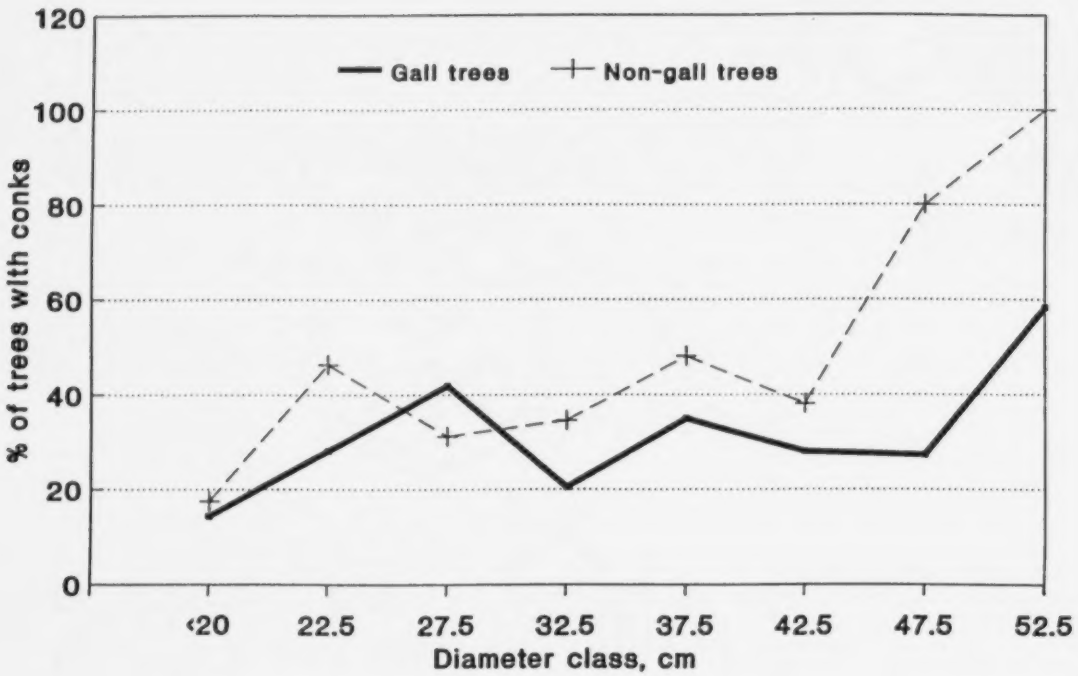


Fig. 5. Percentage of trees in all plots with P. tremulae conks, according to diameter class.

$e^{0.4062}$ or 1.5. In seven of eight diameter classes, gall trees were less likely to have conks than non-gall trees. This consistency is reflected in the absence of a gall-by-diameter interaction and in the near parallelism of the plots of conks vs. diameter for the gall and non-gall trees (Fig. 5).

The presence of black galls may be related to induced systemic resistance against pathogens such as P. tremulae. The development of persistent systemic biochemical changes in response to localized injury or infection by a large number of different agents has been documented, particularly for herbaceous plants. Cucumbers, for example, have been systemically protected against fungal, bacterial, and viral diseases by prior localised infection with Colletotrichum lagenarium (Dean and Kuć 1987). Among the defense chemicals elicited by pathogen attack are low-molecular-weight phytoalexins; hydroxyproline-rich glycoproteins (cell wall components); "pathogenesis-related" proteins, some of unknown function; chitinases and glucanases capable of hydrolyzing pathogen cell walls; and inhibitors of insect gut proteinases (Parsons et al. 1989). Appearance of these substances is usually the result of increased gene expression either within affected tissue or throughout the plant that results in accumulation of defense gene mRNAs and their protein translation products (Bradshaw et al. 1991).

Inducible systemic resistance has been demonstrated in poplars by Bradshaw et al. 1991, Davis et al. 1991, and Parsons et al. (1989), who studied the effects of mechanically wounding lower leaves in poplar to simulate herbivore chewing. They were able to demonstrate a strong systemic response in the form of phytoalexin accumulation in upper leaves. Poplars have at least three different chitinase genes that are systemically

wound-inducible (Davis et al. 1991). It is possible that chemical signals induced by the agent responsible for black gall formation provide a measure of protection against decay organisms such as P. tremulae. Because P. tremulae does become established in some gall trees, the success of this defence is variable and not absolute, perhaps depending on the amounts of defensive chemicals incited, their durability, or speed at which pre-conditioned host trees respond to invasion by decay organisms. Further study is needed to determine whether this variability relates mainly to clonal differences or to environmental factors.

The observed differences in decay susceptibility between gall and non-gall trees might also occur if clones that are genetically prone to gall formation tend also to be superior in resistance responses to decay-causing organisms.

CHAPTER III. ANATOMICAL AND HISTOCHEMICAL COMPARISON OF NORMAL AND GALL WOOD AND BARK

Introduction

The term gall embraces a wide range of neoplastic growths in plants. Galls are pathological structures, ranging from nearly normal to highly complex and abnormal outgrowths, characterized by cellular hypertrophy and hyperplasy (Mani 1964). There have been many attempts to classify galls, mainly according to their morphogenesis. They may be "spontaneous" (internally conditioned) or "induced" (arising from an external agent) (Mani 1964); "kataplasmic" (largely undifferentiated and caused by agents other than insects) or "prosoplasmic" (having characteristic external forms, with well-defined tissues) (Slansky and Rodriguez 1987); "self-limiting" or "non-self-limiting" (Kaiser 1981). None of these terms can yet be applied to black stem galls of aspen.

The lack of information on black galls is probably related to their limited distribution and perceived lack of economic importance. However, given the association of these tumors with reduced stem decay, obtaining basic information on their morphology, anatomy, and etiology can now be more easily justified. Thus an anatomical and histological study was undertaken to compare tumorous and healthy aspen tissue. It was expected that such a study would facilitate comparisons of black galls with published reports of other woody galls and thereby provide a basis for formulating hypotheses about the process of tumorigenesis, such as whether continued growth of gall tissue requires a persistent agent, or whether gall tissue is genetically altered compared with normal tissue.

Methods and materials

Gross morphology and comparison with known poplar galls and stem swellings

Fourteen large stem galls stored at the Northern Forestry Centre were examined in cross section for gross morphology and internal appearance. These galls, collected over 4 yr, had been sawn through with a band saw, sanded on the cut surface, used to obtain isolations of microorganisms, and air-dried. Table 1 lists the sources of these galls, bark thickness, association of galls with branch stubs, wood staining, approximate age of the gall and of the stem at gall level, and any other unusual features. The gross morphology of the mite galls found on aspen stems at Dawson Creek, B.C., was compared with that of each of these 14 galls.

During the field survey part of this study, any unusual swelling on young aspen stems or branches, especially adjacent to gall trees was examined for similarities to large black stem galls. It was hoped that very young stages of black galls might be found. Specimens of the following were examined for gross morphology: Diplodia gall, caused by the fungus Diplodia tumefaciens (Shear) Zalasky; branch galls of the poplar budgall mite, Aceria parapopuli; and galls caused by the flatheaded wood borer, Poecilonota cyanipes (Say). Several swollen branch stubs that did not have typical black gall characteristics were also examined.

Table 1. Gross morphology and estimated ages of black galls examined.

Gall	Source	Tree age, yr	Gall age, yr	Gall diam., cm[a]	Total bark width, cm		Gall origin[b]	Stain[c]	White area[d]		Local dead cambium
					Normal	Gall			Is.	Band	
Likely caused by *A. parapopuli* mites											
1	Hinton	50	14	22	0.4-0.5	0.7-1.7	Diff.	+	+	+	+
2	Whitecourt	70	37	50	0.8-1.1	1.0-3.9	Pt.	+	+	+	+
3	Whitecourt	60	30	22	0.8-0.9	0.7-3.0	Pt.	+	-	+	+
90-2	Hinton	50	30	18	0.4-0.5	0.4-1.3	Diff.	+	+	+	+
90-3	Hinton	55	40	15	0.4	0.7-1.1	Diff.	+	-	+	+
93-2d	Dawson Cr.	40	--[e]	19	0.5-0.6	0.2-2.3	Diff.	+	+	+	+
Cause unknown											
90-1	Hinton	50	33	18	0.6	0.7-1.6	Pt.	+	-	+	+
90-4	Hinton	15	11	5	0.2-0.3	0.5-0.8	Diff.	-	-	+	+
91-3	Hinton	60	30	6	0.3-0.4	0.4-0.9	Pt.	+	+	+	-
91-4	Hinton	70	30	10	0.3	0.3-3.8	Pt.	+	+	+	+
91-5	Blue Ridge	--	40	30	1.0	0.7-3.0	Pt.	++	-	+	+
91-7	Hinton	85	47	16	0.5-0.6	0.7-1.3	Pt.	+	+	+	+
92-1e	Blue Ridge	100	28	11	0.9-1.0	0.7-2.5	Pt.	-	-	+	+
92-3	Ft. Nelson	100	30	16	0.7-0.9	0.7-1.8	Pt.	-	-	+	+

[a]Gall diameter at the widest part, including bark.

[b]Galls either appeared to have a diffuse (diff.) origin at several points in the stem or at a single point (pt.), such as a branch stub.

[c]Presence of stained wood in the non-gall part of the tree is indicated by +, decayed wood by ++.

[d]Presence of white islands (Is.) of tissue within a gall or a band of white xylem tissue next to the cambium is indicated by +.

[e]Could not be determined.

Light microscopy

Because of the extreme hardness of black gall wood, several methods were tried for preparing sections for microscopic observation. A vibrating microtome often shattered the wood, or produced sections of uneven thickness. Blocks for sectioning on a simple freezing microtome were soaked overnight in 10% glycerol, placed on the microtome stage using Cryoform (International Equipment Co., Needham Heights, Mass.) as a matrix, and sectioned at a thickness of 10 or 15 μm. Although satisfactory transverse sections were produced by this method, the curling of longitudinal sections made handling difficult during staining and mounting. Therefore, this method was largely abandoned in favor of the paraffin method. It was also possible to produce satisfactory hand-cut sections of normal wood, but because of abundant dark-staining material in gall wood, hand sections were usually too thick to show cellular detail.

Fixation, dehydration, and paraffin embedding

Four stem galls, not thought to be caused by A. parapopuli, were chosen for detailed anatomical and histological study. Three of these galls are described in Table 1; the fourth (Tree 102) is not listed because samples were taken from the standing tree, and a complete morphological examination was not made. Small galls from upper branches of two trees with multiple stem galls were also studied, as these were thought to be young black galls. Small blocks of bark, cambium, and xylem were removed from cross sections of each gall; comparable blocks of normal-appearing tissues from the same growth rings on the opposite side of each stem piece served as controls. In some cases,

blocks were removed from the apparent gall origin and from patches of white normal-appearing wood (referred to hereafter as "white islands") that were surrounded by darker xylem. Each block was divided into three pieces to provide material for transverse, tangential longitudinal, and radial longitudinal sections. These pieces were no thicker than 3 mm to facilitate infiltration of paraffin (Carlquist 1982), and had a broad face for cutting. This facilitated correct orientation of blocks for microtomy, since the curved grain of the gall wood often made determination of the desired orientation impossible once the blocks were removed from the stem.

Fresh wood samples were chemically fixed in formalin - acetic acid - ethanol (FAA) for at least 1 week, except for one gall, which had been allowed to air-dry, and for which fixation was deemed unnecessary. Because of the hardness of gall wood, blocks were softened in 10% ethylenediamine at room temperature for 3 days (Carlquist 1982; Kukachka 1978) after rinsing with distilled water to remove FAA. They were then dehydrated through a t-butanol series under vacuum and infiltrated with Paraplast X-TRA (Monoject Scientific) under vacuum at 60 C (Jensen 1962). Paraffin blocks were made using a Tissue-Tek II embedding center (Lab-Tek Prod., Westmount, Ill.).

A few sections were cut from paraffin blocks with a rotary microtome to expose the wood, and then the blocks were soaked face down in a small amount of distilled water in a refrigerator for 2 days to enhance successful sectioning by the ethylenediamine method (Carlquist 1982 and personal communication). A thickness of 12 μm was best for producing paraffin ribbons of gall wood. Sections were mounted on microscope slides with Haupt's adhesive (Gurr 1965) before staining.

Histochemistry

Paraffin was removed from sections using xylene, and safranin - fast green staining was used for general anatomical studies, since it provided good cellular definition (Sass 1958; Jensen 1962). Coverslips were permanently mounted with Permount (Fisher Scientific Co.). Photographs were taken on Fujichrome 100 ASA film using a Zeiss Axiophot Photomicroscope under brightfield or phase-contrast illumination.

The following were used as sources for anatomical terminology: Carlquist 1988; Esau 1965, 1977; Martin and Crist 1970; Rees and Shiue 1957-58; Trockenbrodt 1990. These terms are defined in the Appendix.

Unstained paraffin sections of normal and gall wood were dewaxed with two changes of xylene and treated by the following histochemical methods.

Phenolics--Wood sections were tested for presence of phenolics by the Hoepfner-Vorsatz (HV) method (Reeve 1951). Slides were flooded with a mixture of 10% sodium nitrite and 10% acetic acid (1:1). After 3 min, sections were flooded with 2 M sodium hydroxide, rinsed briefly in distilled water, and allowed to dry. A cherry-red or brownish-red color indicated the presence of phenolics.

Lignin and suberin--The location of lignin in wood and bark tissues was determined by the Weisner reaction (Jensen 1962). Sections were dewaxed, rehydrated in an ethanol series, and flooded with a saturated solution of phloroglucinol in 20% HCl. Upon drying, sections developed a non-permanent, deep red-violet color where lignin was present. Slides were dipped in xylene and coverslips mounted with Permount for viewing. Lignin was also detected by its yellowish autofluorescence under epifluorescent

illumination (365-nm excitation and 397-nm barrier filters). However, staining with phloroglucinol - HCl quenched the lignin autofluorescence, allowing the white-blue fluorescence of suberin to be easily seen (Biggs 1987; Rittinger et al. 1987). The presence of suberin was confirmed by staining serial sections of the same tissues as above with Sudan IV. A saturated solution was produced by gently heating Sudan IV in 95% ethanol and glycerin (1:1). The solution was quickly filtered and applied while warm to dewaxed slides. Cover slips were mounted directly over the staining solution. Suberin stained a deep pink to pinkish-orange color.

Calcium oxalate--Wood and bark sections were stained with silver nitrate - dithiooxamide (Yasue 1969; Horner and Zindler-Frank 1982) to determine whether observed crystals were calcium oxalate. Sections were flooded with 5% acetic acid (three changes) to remove calcium carbonate and calcium phosphate, rinsed in double-distilled water, and stained in a 5% aqueous silver nitrate solution for 15 min. After rinsing with water, they were flooded with a saturated aqueous solution of dithiooxamide for 2 min, dehydrated in an ascending alcohol series, and mounted in Permount.

Width of growth rings and cell shape and size

The width of the two youngest growth rings of each gall was measured and compared with the width in normal tissue.

To separate cells for examination, thin strips of xylem were isolated from normal and gall wood from the same growth ring and boiled gently in a mixture of glacial acetic acid : H_2O_2 (1:1) for 2-3 h (Zalasky 1972c). Macerated tissues were rinsed repeatedly in

distilled water, affixed to slides with Haupt's adhesive, and air-dried overnight. Staining in lignin pink (Gurr 1965) for 2 h, dehydration, and mounting in Permount prepared them for examination of cell shape and size. Length and width measurements of vessel elements and libriform fibers included cell walls, length of vessel elements included the "tails," and widths were taken at the widest part of the cell (Carlquist 1988).

Examination for microorganisms, insects, and mites

Because the cause of black galls is unknown, all organisms found in tissue sections were noted. Fungal spores and fruiting bodies were identified when possible. Although fungal hyphae and spores were generally easily seen, especially under phase-contrast microscopy, it was often difficult to determine whether granular material present in and around cells consisted of bacteria. Therefore, three different staining schedules for bacteria were used: (1) thionin - orange G (Gurr 1965), which stains bacteria violet-purple; (2) Giemsa stain in phosphate buffer (pH 6.0) (Wright and Skoric 1928); and (3) toluidine blue O dissolved in phosphate buffer (pH 6.8), in which sections still in wax are directly stained to avoid the damaging effect of alcoholic dehydration on the stain (Preece et al. 1979; Sakai 1973).

Fresh tissue from large galls and several small branch galls was also examined in detail by stereomicroscope, and by light microscope after hand-sectioning and mounting in lactophenol - cotton blue. The presence of insects, mites, bacteria, and fungi was noted.

Scanning electron microscopy

Scanning electron microscopy was used when light microscopy was inadequate to elucidate specific features. Specimens were prepared according to the method of Meylan and Butterfield (1972). Cubes of air-dried wood were boiled in distilled water to soften them, final surface cuts were made with a new razor blade for each surface, and cubes were allowed to dry for several days. They were mounted on aluminum stubs, gold-coated using a Polaron sputter-coater (E5000C-PS3), and examined with a Hitachi S-510 scanning electron microscope.

Results

Macroscopic features of black galls

The major morphological features of the 14 galls examined in cross section are summarized in Table 1. Age determinations apply only to the portion of stem where the gall was located and are estimates only. Trembling aspen is a diffuse-porous wood with latewood almost indistinguishable from early wood. This quality, together with extremely narrow growth rings (<0.5 mm in some samples), made ring counting difficult.

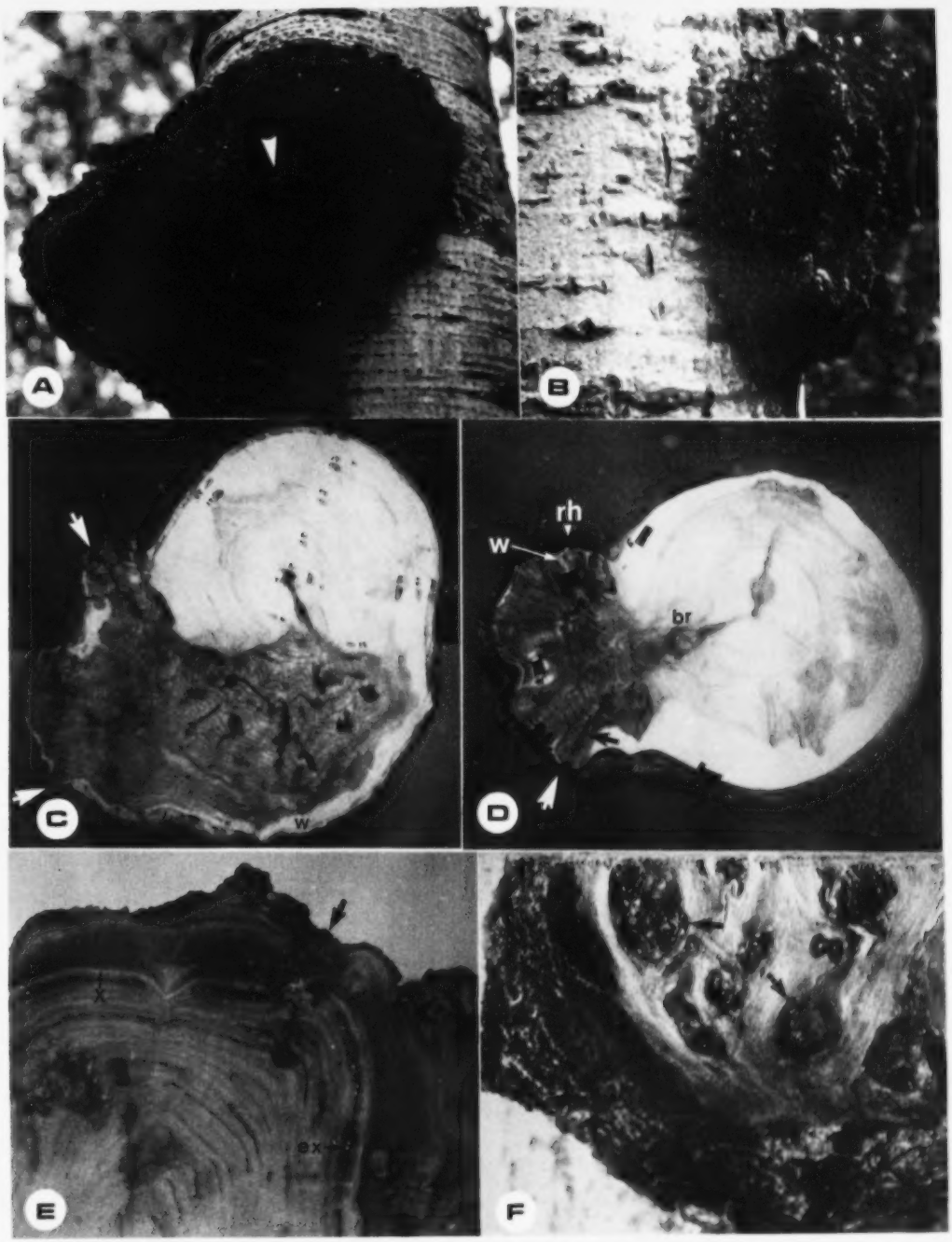
The finding of large active mite galls at Dawson Creek helped to explain some of the morphological diversity seen in previously collected galls. Most of them fell into two categories, those thought to be caused by A. parapopuli and those of unknown cause. The most important difference between mite galls and others was the presence of succulent, deformed green or red buds on the surface of fresh galls or dried buds on older samples. Buds were covered with a thick growth of surface hairs. Living eriophyid mites

consistent with descriptions of A. parapopuli (Wilson and Oldfield 1966) were recovered from both branch and stem galls. Mite galls had an irregular, fine-textured surface, and were somewhat cauliflower-like (Fig. 6A). The rhytidome was thickened over inactive parts of the gall, but not at sites of active bud growth, where this living tissue was in direct contact with the cambial zone, as seen in cross section (Fig. 6C). Sapwood often lacked distinct growth rings and was somewhat darkened compared with normal wood. Mite galls appeared to have originated from many different points around the stem, and different areas had been active at different times. White islands or bands of normal-appearing tissue occurred where mites had not been active for some time, i.e. distinguishable buds were not present on the gall surface. Deep within the sapwood of fresh samples, it was possible to dissect out old buds with characteristic hairy surfaces (Baker and Wharton 1952). This is strong evidence that mites had been resident in these stem galls for many years and were the primary causal agents, not merely secondary invaders. In addition to the Dawson Creek galls, several collected from Hinton and one from Whitecourt were determined to be of this type (Table 1).

In contrast to bud-mite galls, black galls from the plots used in the field study were typically, but not always, globose in shape, with a rough, fissured surface due to thick bark protuberances (Fig. 6B). There was no evidence of either fresh or dried buds on the surface. An elongated area of thickened bark often extended below a gall. Resinous exudates were frequent on the gall surface, appearing to originate in the bark fissures. It was not uncommon to see a broken branch stub embedded in, and extending beyond, a gall. In cross section, the wood of all galls was darkly stained and had an

aromatic, resinous odor. In most cases growth rings were very wide and obvious compared with the normal parts of the stem. In most galls examined, the abnormal growth resulting in a gall had started early in the life of the tree from a single point, often a branch stub. In fact, a darkly stained area often extended to the pith of the stem (Fig. 6D). Most of the stems, outside of the gall tissue, showed a pale to dark yellowish or pinkish stain in the center. One sample was severely decayed by P. tremulae, identified by the presence of a sporophore and typical "punky" heartwood, but the gall itself was sound. All galls had a band of white tissue just inside the cambium, in the most recently formed growth ring (Fig. 6E). In some, this band was much wider, consisting of many growth rings. As in the mite galls, several specimens contained white islands of normal-appearing tissue, where the narrower growth rings caused a sunken area of the gall (Fig. 6D). When cut through within a day of collection, one gall produced a sticky sap exudate along the most recently formed ring of xylem and in small isolated areas of the gall (Fig. 6E). Although normal stem tissue was damp to the touch when fresh, there was no such exudate produced. In the morphological study, 6 of the 14 galls showed similar dried exudates. Although the bark thickness varied over the gall tissue, it was at least two to four times greater than comparable healthy bark as a result of a thick rhytidome (Table 1). Exceptions to this were localized areas where the bark was broken and the cambium appeared dead; these occurred in all but one black gall (Figs. 6D, E). Gall tissue was invariably extremely hard to cut with a scalpel or knife. Most galls, however, contained several radiating strands of crumbly tissue with resin-soaked centers. These strands began in the areas of dead cambium mentioned above (Fig. 6D). When inner and outer bark

Fig. 6. (A) Large stem gall caused by the mite Aceria parapopuli, near Dawson Creek, B.C. Note fine-textured surface and red bud proliferations (arrow). (B) Black stem gall of type found in the study plots. Note coarse bark projections and globose shape. (C) Stem cross section showing interior of mite gall. Gall grew in a diffuse manner around the stem. White band (w) beneath bark indicates area where mites were no longer active. Old buried bud proliferations appear as dark regions within gall. Arrow shows site of recent mite activity. (D) Cross section of black gall from Hinton, Alberta, showing areas used for the microscope study. Note apparent association with branch stub (br), thickened rhytidome (rh), damaged cambium (white arrow) and associated strand of crumbly tissue (black arrow), and islands of white tissue (w). (E) Close-up of black gall showing sap exudate (ex) on cut surface, area of dead cambium (arrow), and thin white band of newly formed xylem (X) inside cambium. (F) Localized dead cambium (arrows) beneath bark of black gall.


A
B
C
W
D
rh
W
br
E
ex
F

were removed, these cambial areas were recognized as raised, localized darkened areas surrounded by creamy-white cambium (Fig. 6F).

Microscopic comparison of normal stem tissue and gall tissue

Tissues are described from the outside of a stem toward the inside, and photographs are arranged in plates according to tissue to facilitate comparison of normal and gall: outer bark (Figs. 7A-F), phloem (Figs. 8A-F), cambium (Figs. 9A-F), and xylem (Figs. 10A-C, 11A-G). Unless otherwise indicated, staining was with safranin - fast green.

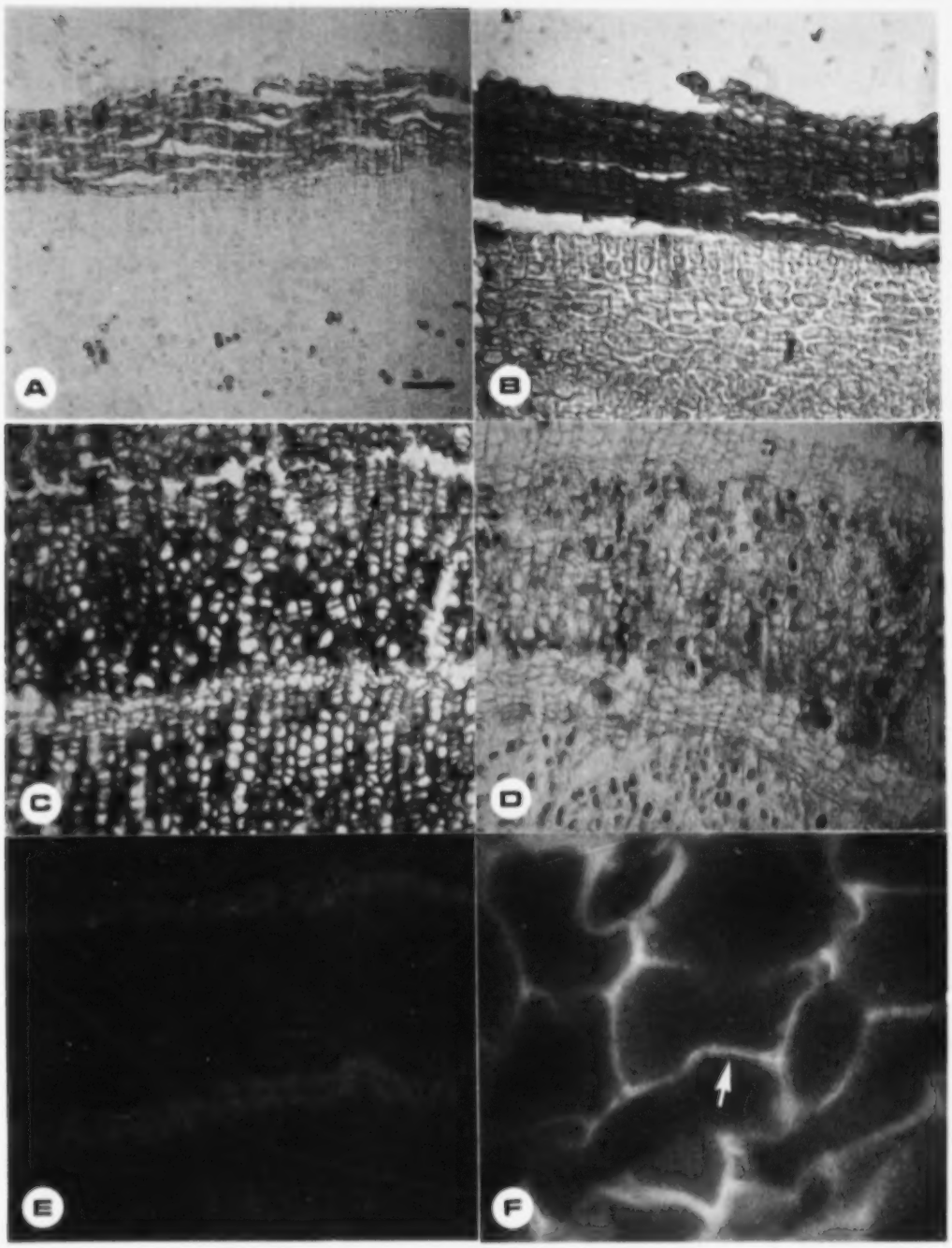
Outer bark

The phloroglucinol - HCl technique to quench lignin autofluorescence selectively and the use of fluorescence to confirm the Sudan IV test were extremely helpful in clarifying the nature of the various layers of aspen rhytidome. Smooth outer bark from the non-gall side of stems consisted of a thin outer periderm. With safranin - fast green, the phellogen was distinguishable as one or two bluish layers inside 10 or more layers of radially flattened cork or phellem cells. Cork cells were heavily impregnated with material that stained positively for phenolics with HV reagents (Fig. 7A). The walls of these cells fluoresced blue-white when stained with phloroglucinol - HCl, and the presence of suberin was confirmed by the Sudan IV stain (Fig. 7B). Phelloderm cells were difficult to distinguish from the underlying cortex. Occasionally a necrophylactic periderm was seen to be walling off localized deeper areas of dead cells, presumably as

a result of local damage or infection. Dark-walled hyphomycetous fungi were invariably present on the bark surface of these areas. One specimen of non-gall outer bark (Tree 102) differed from the others in having a generally thickened rhytidome with several layers of suberized cork cells alternating with narrow layers of lignified, phenolic-containing cells, probably consisting of phelloderm plus cortex cells. The underlying phloem, however, retained the well-ordered arrangement typical of other non-gall samples.

In contrast to the typically thin outer protective layer of normal tissue, galls were covered with a very thick rhytidome (Table 1). It consisted of alternating layers of cells, as noted above for normal bark of Tree 102. However, in gall outer bark, the layers containing highly lignified cells (Fig. 7C) were much wider than in "normal" thickened bark of Tree 102; these cells were heavily occluded with phenolic deposits, as shown by HV staining (Fig. 7D). Druse-like crystals were occasionally found within these layers. Alternating with these layers were narrow bands of thin-walled, rectangular cork cells that appeared empty. Blue-white fluorescence of phloroglucinol-stained material indicated that their walls were lined with a suberized layer (Figs. 7E, F). Dematiaceous hyphomycetes were abundant on the outer bark surface of all galls examined, and hyphae were occasionally seen deeper within the bark, especially within these suberized layers. The most recently formed suberized layer separated the rhytidome from the underlying secondary phloem.

Fig. 7. Aspen outer bark, TS. (A, B) Normal bark showing sloughing phellem cells of exophylactic periderm stained for (A) phenolics (HV stain) and (B) suberin (Sudan IV). Underlying phelloderm, cortex, and phloem cells also contain phenolic substances. (C - F) Thickened rhytidome of black gall showing (C) heavily lignified dead phloem (red, phloroglucinol - HCl stain) cut off by successive necrophylactic periderm layers (np), (D) phenolic substances in the same layers (orange-brown, HV stain), (E) blue-white fluorescence of suberized cork cells, and (F) greater magnification of (E), showing suberin deposited inside phellem cell walls (arrow). Scale bar: A, C-E, 100 μm; B, 50 μm; F, 10 μm.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Phloem

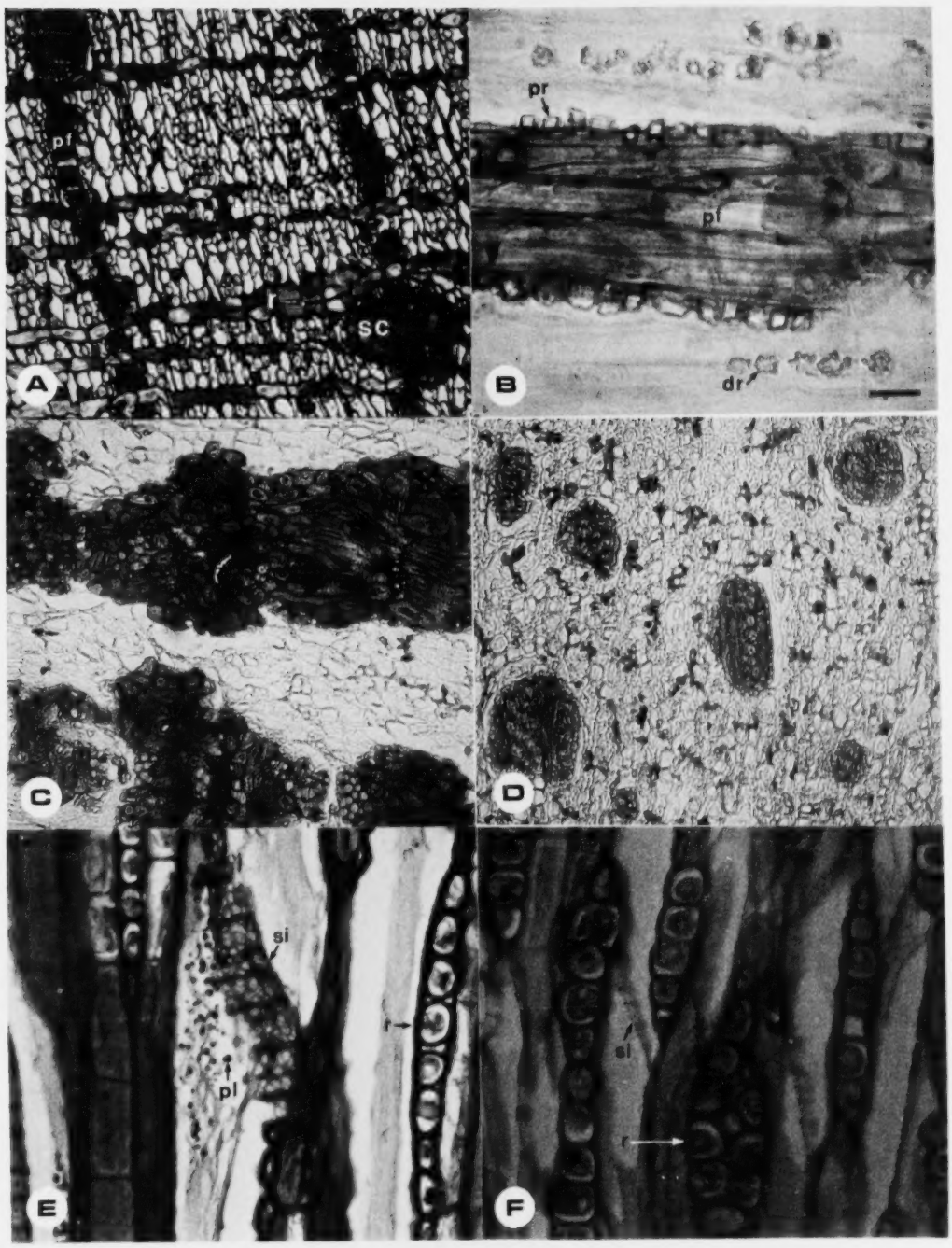
The inner bark of normal stem pieces (Fig. 8A) consisted of a progression of cells from young phloem near the cambium, to older phloem exterior to this, and to cortex inside the outer periderm. Phloem elements (sieve tube members, companion cells, phloem parenchyma) were easily distinguishable and occupied a large part of the bark, with their frequency decreasing towards the outside of the stem. Phloem rays could be traced in long continuous lines from the cambium to the cortex. For most of this distance they were uniseriate, but close to the outer bark their width increased considerably, due both to cell expansion and anticlinal divisions to accommodate radial growth of the stem. The distinction between the oval cells of the cortex, just inside the periderm, and these expanding ray cells was not obvious. Rees and Shiue (1957-58) state that the cortex of aspen does not contain fibers, so this was used to distinguish between phloem and cortex. The brightly staining fibers of older phloem were a striking feature of inner bark. In transverse section, these very thick-walled, heavily lignified cells occurred in regular bands two or three cells wide across the secondary phloem and parallel to the bark surface (Fig. 8A). It is not known whether these bands delimit yearly phloem increments, as has been determined for apple (Evert 1963). In longitudinal section, fibers were long, straight cells with pointed ends. Phloem elements sometimes appeared collapsed immediately inside of these fiber bands, perhaps as a result of secondary development of fiber walls. In transverse sections of the oldest stems, the rows of fibers were broken only by phloem rays traversing them and by bundles of sclereids, which became more frequent towards the outside of the stem. The irregularly shaped sclereids had

polylamellate walls, simple pits, and very reduced lumina, often occluded by phenolic substances (Fig. 8C). Because of their position, the sclereids often appeared to have differentiated from the cells of the dilated rays.

In longitudinal view, oblique end-walls of sieve tube members were covered with well-developed compound sieve plates. In early stages of differentiation, sieve pores were occluded by a safranin-positive substance, which had disappeared in mature sieve tube members. It was assumed that the numerous disc-shaped bodies observed in immature sieve tube members were starch plastids (Esau 1965) (Fig. 8E). A single large red-staining body, probably an extruded nucleolus, and an irregularly shaped slime plug, probably consisting of P-protein, which accumulates on sieve plates in response to injury, were also characteristic of differentiating sieve tube members (Esau 1965, 1977; Evert 1963). Two types of intracellular crystals were very abundant throughout the phloem: prismatic crystals, one per cell, in vertical parenchyma surrounding phloem fibers and less frequently around sclereids; and irregular, druse-like crystals, one to three per cell, in axial phloem parenchyma (Fig. 8B). Both types stained dark brown to black with silver nitrate - dithiooxamide, a positive test for calcium oxalate. Druse crystals stained especially darkly.

Young gall phloem seen in tangential longitudinal section contained many fewer and much smaller phloem sieve tubes than did normal tissue. As in normal developing sieve tube members, round starch plastids were present in the cell lumina, but they were small and less abundant. The sieve plates of these cells were fragile looking, and pores were small (Fig. 8F). Bundles of both sclereids and lignified phloem fibers were seen

Fig. 8. (A) Normal phloem (TS), showing regular bands of phloem fibers (pf) perpendicular to phloem rays (r), and a bundle of sclereids (sc). (B) Phloem fibers (RLS), healthy tissue, showing calcium oxalate crystals in prismatic (pr) form associated with fiber bundle (pf), and in druse (dr) form in other parenchyma cells (lignin pink stain). (C, D) Well-developed sclereid groups in normal phloem (C) and smaller bundles in gall phloem (D) (TS, phloroglucinol - HCl). (E, F) TLS of recently differentiated phloem in normal (E) and gall tissue (F). Note abundant starch plastids (pl), well-developed sieve plate (si), and uniseriate ray (r) in normal tissue; in gall phloem, sieve plates look fragile, starch plastids are infrequent, and rays are multiseriate. Scale bar: A, C, D, 100 μm; B, E, F, 25 μm.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very close to the cambial area, and were well-lignified before phloem elements had differentiated (Fig. 9C). These sclereids and fibers, however, were in much smaller bundles, and were scattered erratically throughout the phloem (Fig. 8D), rather than in rows as in normal wood (Fig. 8C). It was often difficult to distinguish phloem rays from surrounding tissues, since much of the phloem consisted of undifferentiated parenchyma rather than well-defined phloem elements.

Phenolic compounds occurred throughout the cortex and phloem of both normal and gall samples, with amounts generally being greater in gall cells. In the cortex and phloem parenchyma, phenolic compounds appeared to encrust the inner cell walls; they were also abundant in ray parenchyma and in lumina of sclereids (Table 2). Calcium oxalate crystals were common throughout gall phloem, as in normal samples, although a somewhat greater frequency of druse-containing parenchyma may reflect a proliferation of parenchymatous cells rather than axial elements, with which prismatic crystals are associated.

Cambium

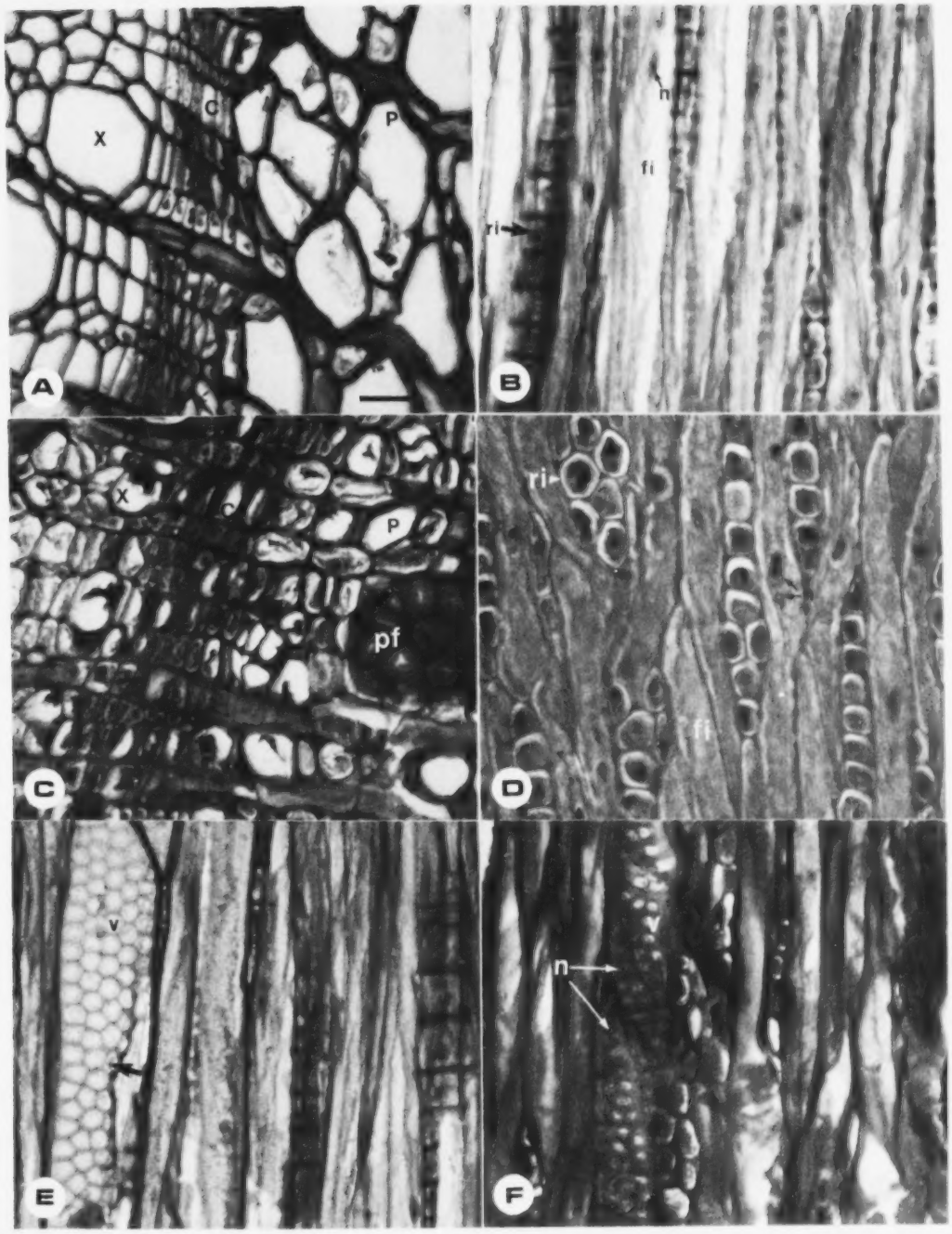
Because of the difficulty of distinguishing a single row of cambium initials from their recently produced derivatives, the term "cambium" will be used synonymously with "cambial zone" (Evert 1963; Esau 1965).

Regardless of collection date, normal cambium was easily discerned in transverse section as a band consisting of six to nine rows of radially flattened cells (Fig. 9A). Depressed pit fields gave the walls a beaded appearance in tangential view. Fusiform

initials were generally very long and had overlapping ends, characteristic of a nonstratified cambium (Evert 1963). Undifferentiated cambial cells were readily distinguished from phloem and xylem initials by their dense, uniformly distributed cytoplasm, prominent nuclei, and very thin or absent cell walls (Fig. 9B). Nuclei consisted of pale, rounded or elongated nuclear envelopes containing granular material and round, bright-red-staining chromatin. Often two nuclei were seen side by side, suggesting that mitosis had recently occurred, but cell walls had not yet formed. Surprisingly, mitotic figures were not seen in any of the samples. Although collections were made at various times during the field season, it was not possible, because of the small number of samples originating from several locations, to draw definite conclusions regarding the normal duration of cambial activity in aspen. Partially differentiated phloem and xylem initials (Figs. 9A, E), i.e. xylem vessels and fibers without secondary walls and phloem sieve tube members containing slime plugs (Evert 1963), were seen adjacent to cambium in all samples. Even in a sample collected in October, xylem elements immediately adjacent to the cambium still retained some cytoplasm, and fusiform initials sometimes contained more than one nucleus, with cell plates not formed or only partially formed between them. Recently divided nuclei, however, were much more abundant in a sample collected in June.

The walls of gall cambium in transverse section were indistinct (Fig. 9C), making it difficult to determine how many cell layers made up this tissue. In a sample collected in June, the cambium was at least 11 cells wide, and young xylem elements still retained cytoplasm 14 rows away from the cambium, a much greater distance than in healthy

Fig. 9. (A, B) Normal cambium. (A) TS, cambial cells (C) are in well-ordered rows, and xylem (X) and phloem (P) derivatives differentiate soon after cell division. Phloem fiber bundles are seldom seen close to cambium. (B) TLS. Walls have not yet formed around small, recently divided nuclei (n) (ri, ray initials; fi, fusiform initials). (C, D) Gall cambium. (C) TS, cambial initials (C) appear crowded and disorganized; most phloem (P) and xylem (X) derivatives are not well-differentiated near cambium, except phloem fiber bundle (pf). (D) TLS. Many dividing nuclei occur in close proximity; rays are short with large rounded cells, and they are becoming multiseriate. (E, F) TLS. Xylem initials of normal (E) and gall (F). (E) By the time vessel (v) walls have become pitted (arrow), nuclei are no longer seen. (F) Gall vessels still retain nuclei when bordered pits are forming. Note shortness of vessel elements. Scale bar: 25 μm.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tissue. Xylem initials were crowded, and appeared parenchyma-like and undifferentiated (Fig. 9C), unlike normal tissue (Fig. 9A). In tangential section, fusiform initials in the cambial zone were much shorter than normal and their orientation was distorted by wide multiseriate rays. There appeared to be many nuclei of variable size and shape in each cell (Fig. 9D). The chromatin bodies in recently divided nuclei were often considerably larger than in comparable normal tissue. The general impression was of rapid cell division, causing cell crowding and grain distortion, fewer recognizable cells, and cell maturation occurring further from the cambium than normal. As an example of the longer juvenile state of differentiating gall tissue, xylem vessel members still retained their nuclei at the time pit fields became visible on their side walls (Fig. 9F); nuclei were never seen in normal vessel elements at this stage (Fig. 9E).

The occurrence of phenolic compounds in cambial tissue was variable, from very little detected in normal samples to a high concentration in one gall sample collected in July (Table 2).

Xylem

Healthy aspen sapwood consisted almost exclusively of libriform fibers, vessel elements, and ray parenchyma (Figs. 10A, 11C). In this diffuse-porous species the vessels appeared singly or in small groups in transverse section and decreased only slightly in pore diameter from early to late wood. Vessels were straight, open tubes having lateral pit fields of round to oval bordered pits where they were in contact with other vessels, groups of large simple pits where ray cells crossed them, and a simple

perforation at each end where one vessel element joined another to form water-conducting vessels. A short tail extended longitudinally beyond end-wall perforations. Occluded vessels were not seen in any of the normal sapwood tissues examined. Rays were uniseriate and homocellular. Even in older tissue (12-17 years), ray parenchyma cells retained a nucleus and cytoplasm, and were presumed to be living. Libriform fibers had pointed ends and thick walls with a few inconspicuous simple pits; it is not certain whether they were entirely devoid of cytoplasm, because a fine granular material was sometimes seen in fibers of macerated tissue. Vertical parenchyma was of the boundary apotracheal type and was infrequent, seen only in radial section as one or two narrow rows in the latewood separating adjacent growth rings. These microscopic observations of aspen sapwood are consistent with descriptions given by Kennedy (1968), Wilson and White (1968), and Panshin and de Zeeuw (1970).

Lignin of healthy sapwood was most obvious in the middle lamellae between cells, as detected by both safranin and phloroglucinol - HCl staining. With the exception of small granules of phenolics in normal ray cells of one sample, these compounds were not detectable by HV staining in normal xylem (Table 2). Similarly, calcium oxalate crystals were rare.

Yearly growth increments in gall tissue were two to three times wider than normal rings produced the same year. However, this dramatic difference was not true of one sample, which had a broad white band of normal-appearing tissue just inside the cambium (BG-91-3, Table 3).

Table 2. Quantitative assessment of phenolic compounds in various stem tissues of normal (N) and gall (G) wood and bark, based on staining by the Hoepfner-Vorsatz method.[a]

	Bark						Xylem			
Gall	Rhytidome	Sclereid lumina	Cortex walls	Young phloem	Old[b] phloem	Cambium	Rays	Fibers	Vessels	Callus
BG-91-3										
N	++++	++	++	-	+	-	++	+	-	NA
G	+++/++++	++	+++	+	+/++	-	+/+++	+	-	NA
BG-91-7[b]										
N, young	++++	-	++	-/+	+	-	-	-	-	NA
N, old	NA	NA	NA	NA	NA	NA	-	-	-	NA
G, young	++++	+++	+++	+	+++	NA	-/+++	-/+	-/+	NA
G, old	NA	NA	NA	NA	NA	NA	-/+++	-/+++	-/+	-/+
G, island	NA	NA	NA	NA	NA	NA	+/++	-/+	-	NA
BG-92-1e										
N	++	-/+	-/+	-/+	-/+	-	-	-	-	NA
G	++++	++	+++	++	++	+	+/+++	-/+++	-/+++	++
Inf. pt.	++/++++	++/++++	++/++++	NA	NA	+	+/++++	+/++++	+/++++	++/++++
Tree 102										
N	+++/++++	+	+	-/+	+	+	-	-	-	NA
G	+++/++++	+	+	++	++/+++	++/+++	+/++++	+/++++	+	++/+++

[a]Explanation of symbols: - phenolics absent; + small particles in some cells or small amount in walls; ++ a few cells full, or particles in many cells; +++ solid mass, half-filling cells; ++++ solid mass filling most cells; NA, not applicable or not available; inf. pt., possible infection point (dead cambium). An oblique line between symbols indicates a range within a given tissue.

[b]Young means tissue sample take near the cambium; old, outer parts of phloem, and xylem 12-17 yr old.

Table 3. Comparison of mean cell size[a], mean ray height (from 10 rays), and width of annual growth rings from normal (N) and gall (G) xylem. Because cell size varies with tree age, comparisons should only be made between normal and gall tissue within a given sample (Kennedy 1968).

	Fibers		Vessel elements			
	Length, µm	Width, µm	Length, µm	Width, µm	Ray ht., cells	Ring width[b], mm
BG-91-3 (May)[c]						
N	695 a	23 a	527 a	66 a	--[d]	0.07 (2nd)
G	652 b	23 a	486 b	48 b	--	0.09 (2nd)
BG-91-7 (October)						
N	949 a	22 a	685 a	95 a	14.4 a	0.4 (1st)
G	381 b	23 a	194 b	60 b	8.7 b	1.4 (1st)
Is.[e]	715 c	22 a	434 c	80 c	8.5 b	--
BG-92-1e (June)						
N	931 a	21 a	622 a	91 a	10 a	0.2 (1st)
						0.6 (2nd)
G	347 b	21 a	176 b	76 b	6 b	0.7 (1st)
						1.9 (2nd)
Tree 102 (July)						
N	1025 a	24 a	618 a	95 a	10.5 a	0.35 (1st)
G	392 b	24 a	152 b	61 b	4.9 b	0.78 (1st)

[a]At least 10 vessel elements and 20 fibers were measured to obtain the means for cell size. Means within the same column and same tree are significantly different ($P<0.05$) if followed by different letters.

[b]Because some galls were collected in the spring before the current year's growth ring (1st) was complete, increment widths are also given for the previous year (2nd).

[c]Gall number and month of collection.

[d]A dash indicates data not available.

[e]White island within the gall.

Throughout most gall sapwood, the expected cell types were easily recognizable (Fig. 10B). However, in transverse section it was obvious that the ratio of fibers to vessels was much greater than normal. Cell measurements from macerated xylem showed that gall fibers and vessel elements from three of four samples were considerably shorter than in healthy tissue, and that vessels were unusually small in diameter (Table 3). Vessel elements were seen to have unusual shapes, often small and barrel-like, with perforations lateral as often as terminal. Fibers often had twisted ends and contained a granular substance (Fig. 11D). Rays were multiseriate, shorter than normal (Table 3), and consisted of cells that were abnormally wide and round in tangential view.

Severe disruption of cell orientation was evident throughout gall xylem. In transverse section, it was common to see abrupt changes from transverse to longitudinal appearance of cells. In radial view, grain was wavy; in tangential section, fibers and vessel elements were often in circular whorls around groups of ray cells (Fig. 10C).

Gall xylem was characterized by several unique intracellular features that were not seen in normal sapwood. Vessels were frequently criss-crossed by tylosis walls, and often contained a grey, granular or fibrous substance (Fig. 10B). It was sometimes possible to see small tyloses ballooning through the simple pits from adjacent ray parenchyma (Fig. 11B). Tyloses sometimes had secondary wall thickening, and a nucleus was occasionally seen in recently formed ones. Coincident with tyloses in vessel elements was the frequent presence of HV-positive substances in ray cells and fibers. Ray cells were often completely occluded by these solid substances. Both tyloses and phenolic deposits became more frequent with tissue age. They were obvious in 1-year-old cells, and could

Fig. 10. (A) Normal xylem of aspen (RLS) showing regular vertical arrangement of libriform fibers (lf) and vessel elements (v), and part of a uniseriate ray (r). Cells are clear and unoccluded. (B) Gall xylem (RLS), same tree as (A). Vessels are filled with tyloses (t) and granular material (g); fibers are small and unusually numerous; ray cells are completely occluded by dark deposits. (C) Gall xylem (TLS) showing whorled arrangement of axial elements around ray cells. Scale bar: A, B, 50 μm; C, 25 μm.

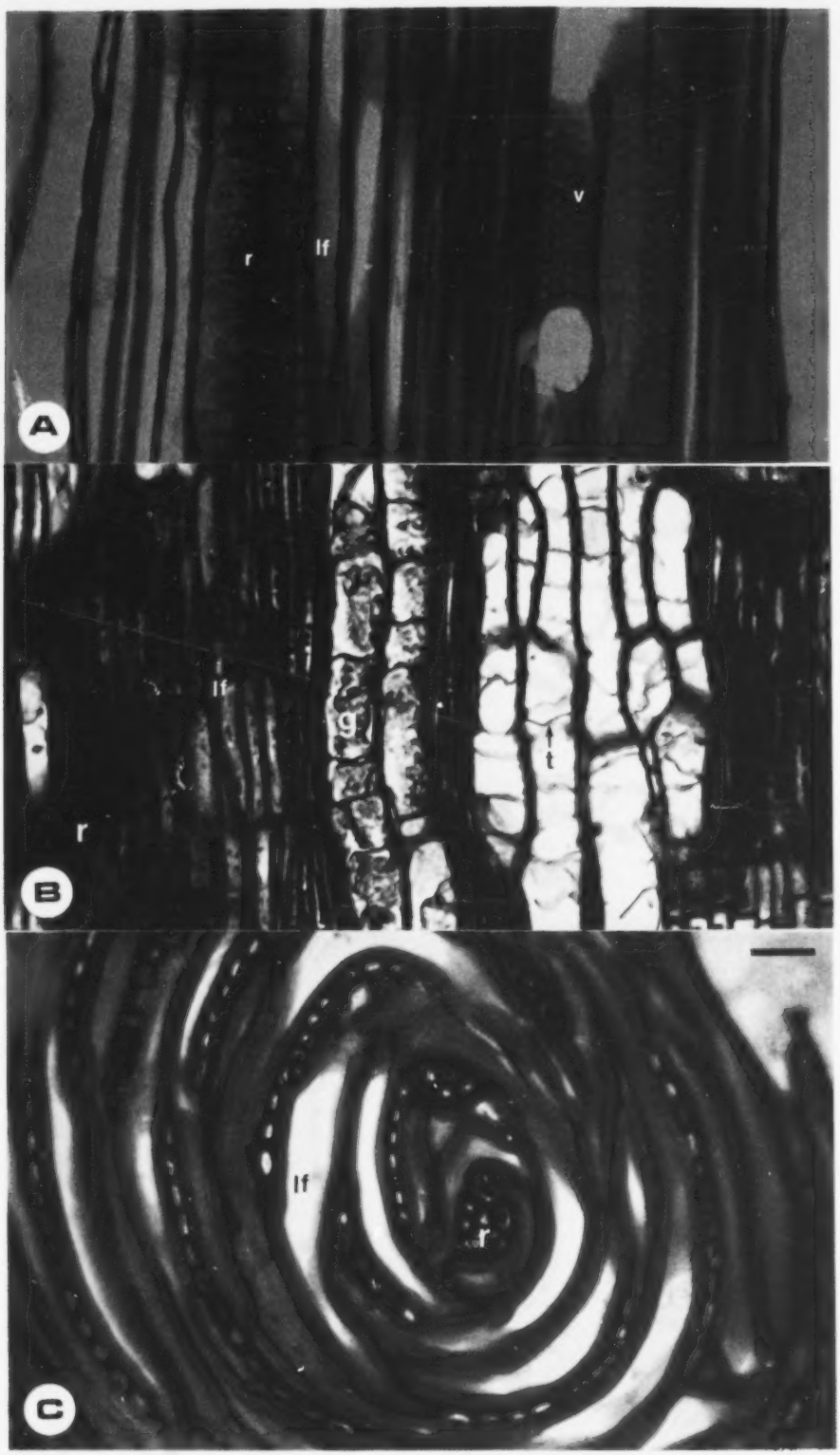
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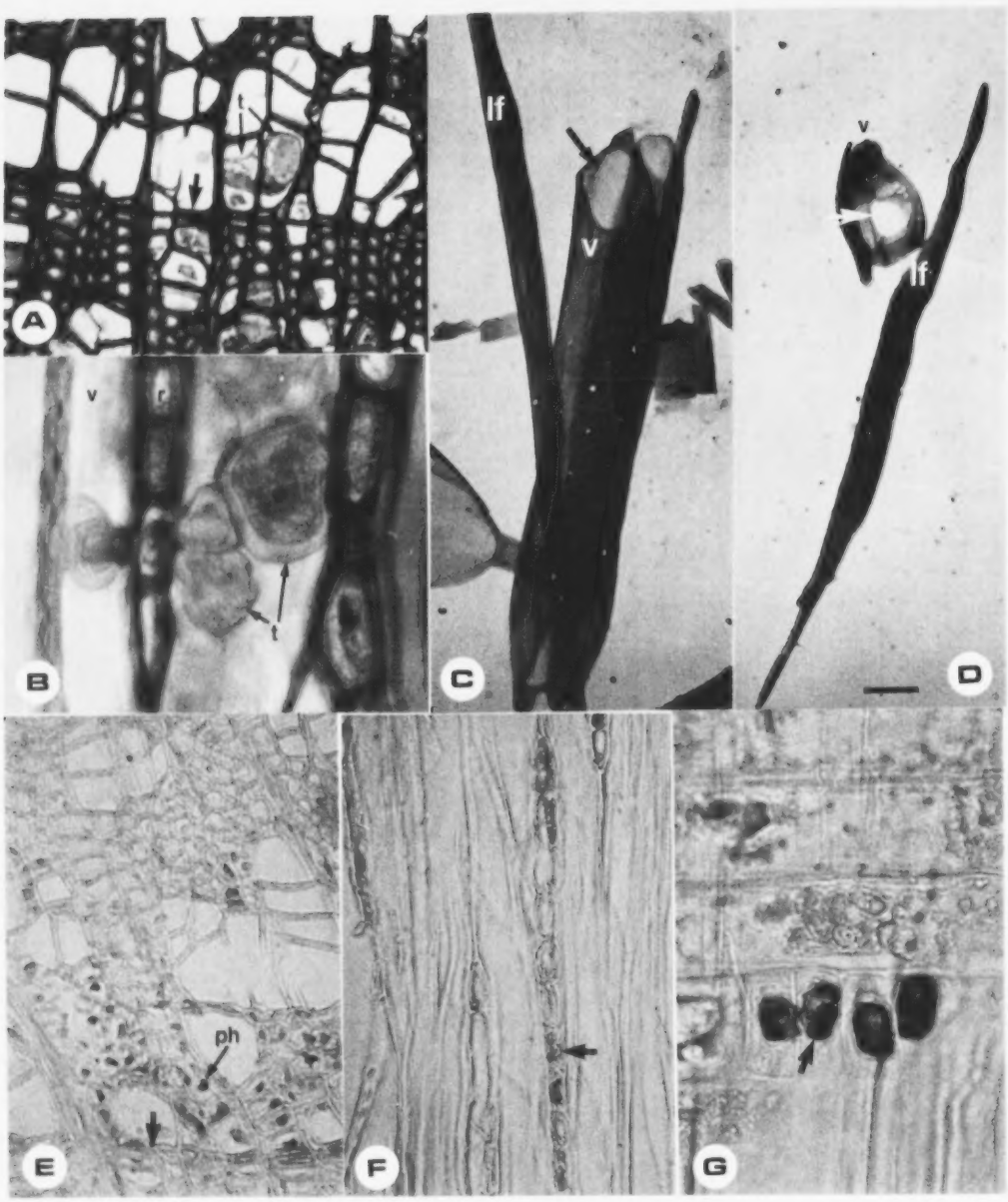
Fig. 11. (A) Tyloses (t) forming in vessels of gall xylem at end of current growth ring (TS) (large arrow marks limit of most recent growth ring; cambium would be towards the top); vessels of previous year (bottom) contain many tyloses. (B) Ballooning of cytoplasm from ray parenchyma into vessel to form tyloses (TLS). (C, D) Xylem cells after maceration of normal wood (C) and gall wood (D). Note abnormal shape and size of gall vessel, shortening and twisting of fiber, and granular contents in fiber (arrows indicate perforations). (E) Occlusion of many cells of gall xylem with phenolic compounds (ph) in oldest part of most recent growth ring (TS, HV stain) (large arrow marks limit of growth ring; cambium would be towards the top). (F) Xylem of gall island, showing normal appearance of cells, but small phenolic inclusions in ray parenchyma (arrow) (TLS, HV stain). (G) Prismatic calcium oxalate crystals (arrow) in vertical ray parenchyma of gall xylem (silver nitrate - dithiooxamide). Lighter inclusions in cells above crystals are phenolic-filled vacuoles. Scale bar: A, C-E, 50 μm; B, G, 10 μm; F, 25 μm.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usually be seen in the oldest part of the current year's growth increment, depending on the time of collection (Figs. 11A, E). There were sometimes calcium oxalate crystals of the prismatic kind in vertical parenchyma separating growth rings or in ray parenchyma (Fig. 11G). No change from normal was seen in the position of lignin deposits, but middle lamellae between cells tended to be wider, and cell walls seen in radial section stained more intensely for lignin, giving the overall impression of greater lignification in gall xylem tissue. All of these characteristics combined would give gall tissue the darkly stained appearance noted macroscopically.

White islands--White islands of xylem within surrounding dark gall tissue appeared to be normal in most respects. Fibers and vessel elements showed the orderly arrangement typical of healthy aspen, vessels were free of tyloses and granular deposits, and rays were uniseriate (Fig. 11F). However, length of fibers and vessel elements was intermediate between gall and normal xylem, whereas number of cells per ray was more typical of gall tissue than of normal (Table 3). Small particles, which stained positively for phenolics, were present in ray cells (Fig. 11F). These particles were similar to those seen in one normal tissue sample, but much less than in typical gall tissue (Table 2).

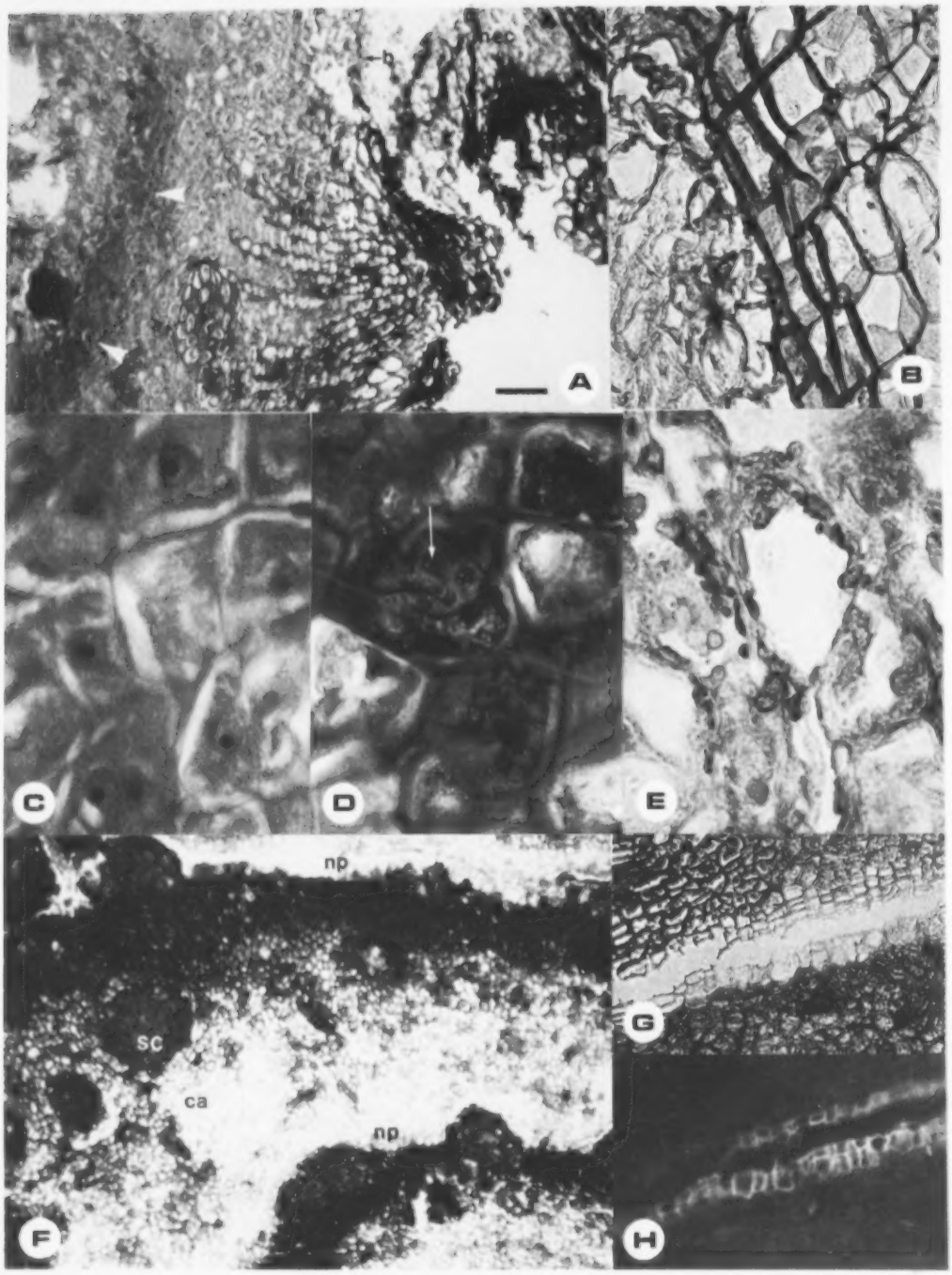
Wood callus and possible infection points--As mentioned previously, a recognizable, although somewhat abnormal, cambium was present over most gall sapwood. At irregular points, however, the cambium appeared to be dead. Transverse sections through these areas showed a definite break or wound, bordered by a mass of parenchymatous or callus cells. Within this callus was a distinguishable band of rapidly dividing cells with obvious nuclei (Figs. 12A, C). This band was continuous with the

intact cambium, but near the necrotic area it extended upwards toward the rhytidome (Fig. 12A). Because the many cells of this callus region contained nuclei and cytoplasm, they were assumed to be living when sampled. However, toward the outer edge of this zone, near the dead tissue, many cells were blackened and plasmolyzed, and round droplets, which may have been phenolic-containing vacuoles, were frequent in the cytoplasm (Fig. 12D). An abrupt border on the outer edge of the callus zone marked the beginning of several layers of thin-walled, suberized cells (Fig. 12B) that appeared empty. The very center of the dead zone consisted of masses of crushed dead cells that were devoid of contents except for phenolic droplets on inner walls (Fig. 12E).

Within the callus zone, but further away from the dead region, isolated groups of recognizable phloem sieve tubes and xylem vessels were evident, with numbers of differentiated cells increasing with distance from the dead area.

Crumbly areas of tissue were macroscopically visible within the xylem of all galls except one (Table 1). These tissues were continuous with the areas of dead cambium described above. Microscopic observation showed that these crumbly areas consisted largely of crushed degraded cells surrounded by parenchymatous tissue within the xylem (Fig. 12F). They were somewhat similar to the rhytidome of black galls in having layers of highly lignified, phenolic-containing cells alternating with thin-walled, empty suberized layers (Figs. 12G, H). Along the outer edge of the lignified layers were groups of thick-walled sclereids with accompanying crystalliferous cells. Sclereids were not seen in healthy aspen sapwood. At the border of the callused area, cells gradually became more recognizable as xylem elements, albeit deformed.

Fig. 12. (A) Region of cambial damage in gall (TS), showing broad band of rapidly dividing callus cells (arrows) extending upward from intact cambium to rhytidome, adjacent dying cells (d), and well-defined border (b) between living and dead cells (nec). (B-E) Details of (A), showing (B) suberization of cells bordering dead region (orange-red, Sudan IV); (C) rapidly dividing callus cells; (D) plasmolyzed cells that appear to be dying, with possible vacuoles containing phenolics (arrow); (E) cell in necrotic region devoid of contents except for phenolic droplets. (F-H) Wood callus region in old (>12 yr) xylem of gall (TS). (F) Degraded center (not shown, but would be at lower right) is surrounded by bands of undifferentiated callus (ca) and heavily lignified, phenolic-containing cells (red) walled off from rest of xylem by necrophylactic periderms (np). Groups of thick-walled sclereids (sc) are common. (G) Detail of lignified cells (red with phloroglucinol - HCl) from top of (F). (H) Same as (G), but with fluorescence microscopy to show suberized nature of cork cells of necrophylactic periderm surrounding wood callus. Scale bar: A, F, 100 μm; B, 25 μm; C-E, 10 μm; G, H, 50 μm.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An "atypical" black gall

After the microscopic examination of large stem galls, it was concluded that BG-91-3 differed in several features from the other stem galls examined. The white band next to the cambium was unusually wide, comprising several growth rings; there were no localized areas of dead cambium or crumbly regions within the xylem; cell size and shape were closer to normal than in other galls; and the bark was greyish and less rough than the other samples examined. However, in older regions of the gall, darkly stained xylem with tyloses in vessels and large quantities of phenolics in ray cells, hyperplasia and accompanying cell whorls and wide growth rings, more closely resembled the other stem galls examined.

Search for young galls

The results of examinations of small galls found on aspen during the field study included several galls of known cause and others of unknown cause. They are described below.

Insect galls--Spindle-shaped galls on stems of saplings near Blue Ridge were determined to be insect-related because of obvious galleries within. Although larvae were not found, the probable cause was the poplar gall borer, Saperda populnea moesta LeConte. Blackened, irregular branch and stem galls collected at Edmonton contained galleries and larvae of the flatheaded wood borer, Poecilonota cyanipes (Ives and Wong 1988). Although insect galleries were seen in one of the black stem galls examined, they also occurred elsewhere in that tree stem.

Diplodia galls--Small globose stem galls collected near Blue Ridge and in Elk Island Park on young aspen were obviously caused by Diplodia tumefaciens. Characteristic black pycnidia were embedded in outer bark (Hiratsuka 1987). Black perithecia of Cucurbitaria staphula Dearness ex R.H. Arnold & R.C. Russell (Arnold and Russell 1960) and apothecia of the discomycete Leciographa gallicola Funk (Funk 1979), common associates of D. tumefaciens, were frequently present on Diplodia galls. The main cause of swelling in these galls was abnormal thickening of phloem tissue; xylem browning similar to that found in black galls was absent. Diplodia tumefaciens was found on only one black stem gall, where it occurred on the edge of a bark fissure.

Branch and twig swellings on gall trees--Two heavily galled trees from Blue Ridge yielded the most likely young black galls found so far. In addition to 10 or more large main-stem galls, a gradation of many smaller galls and swellings was present in upper branches. Small twigs, in fact, had a gnarled appearance (Fig. 13A). Similar swellings were not seen in branches of felled, non-gall trees from the same plot. Neither were they seen in the upper branches of trees from Dawson Creek with large mite-caused stem galls, although these trees bore typical mite galls on some upper branches. At their smallest distinguishable stage (<1 cm across), swellings had smooth bark, but in larger branch galls, bark was rough, broken, and blackish at the top. In fresh samples, the tissue underneath this rough surface was soft and brown (Fig. 13B). Browning extended inward to the cambium and was connected to brown xylem tissue. In paraffin sections cut successively downward through a gall, parallel to the twig axis, the center of this area appeared degraded or was completely empty; surrounding cells were full of phenolic

droplets (Fig. 13C). In sections cut perpendicular to the twig axis, this soft brown area was shown to consist of a distinct tissue extending from the outer gall surface through a disrupted cambium. Parenchymatous cells were arranged in layers of lignified pitted cells alternating with empty cells with suberized walls, a situation similar to that of wood callus and disrupted cambium in large stem galls. This tissue was well demarcated along the margins and separated from living phloem, cambium, and xylem by cells that stained strongly for suberin with Sudan IV. Beyond this barrier, tissues appeared relatively normal, except that phloem was unusually thick. Secondary areas of degraded cells often also extended inward from the outer bark at the sides of a gall; these were also surrounded by well-developed periderms (Fig. 13D).

Towards the center of the twig, and connected with the main damaged area, high concentrations of phenolics completely filled the cells around the pith. Xylem vessels near the darkened center of twigs contained tyloses. Twig centers contained dead, degraded cells interspersed with dark-walled fungal hyphae and spores (Fig. 13E). Sclerified cells were also frequent in the center, along with dense bundles of smaller-diameter, thick-walled lignified fibers. From successive sections parallel to the twig axis, it was seen that these bundles were continuous from the center of the twig to the outside, and it was concluded that they were the remains of leaf traces. Near the exterior of the twig, these fibers were surrounded by protective periderm layers. Fungal fruiting bodies and dark spores were usually present at the bark surface of these leaf traces, and sometimes within them.

Fig. 13. (A-E) Knob-like branch galls found on two trees with multiple stem galls, Blue Ridge. (A) A continuous gradation of gall sizes from <1 cm to large stem galls were found on each tree (ruler = 15 cm). (B) Top cut off branch gall, showing brown region, which is continuous from broken surface at top of gall inward to pith. Small fruiting body at left (arrow) is of Phaeocalicium populneum. Gall was about 1 cm across. (C) Detail of brown area (toluidine blue stain). Center is empty or with some cell fragments. Dead cells contain droplets, possibly phenolic compounds (ph), similar to those seen in the dead cambial region of stem galls (Fig. 12E). A well-defined cork layer (arrow at top) separates the necrotic region from living parenchymatous tissue and, deeper within gall, from deformed xylem. (D) Necrotic region extending from the side of the gall into the cortex. A thick cork layer (arrow) separates it from living tissue. (E) Degraded center of twig below small gall. At higher magnification, dark-walled fungal hyphae and spores are seen in this region. For C-E, scale bar = 100 μm.

A
B
C
ph
D
E

Presence of microorganisms

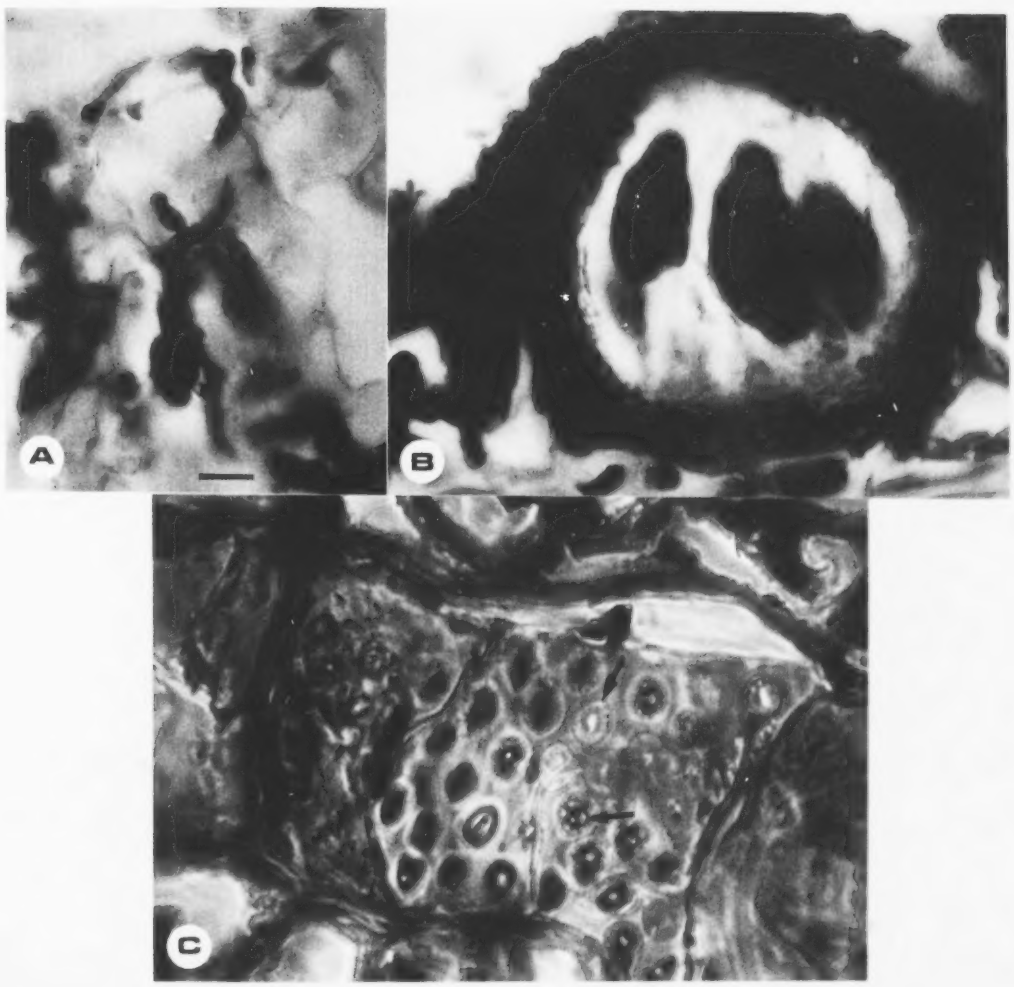
Dematiaceous hyphomycetes were the most commonly encountered microorganisms on black galls (Fig. 14A)--on the outer bark of all stem galls, on the surface of leaf traces and thickened rhytidome of small branch galls, and where thickened rhytidome occurred in isolated patches on normal stem sections. These fungi were believed to be a mixture of species, but Xylohypha lignicola Sutton (1973) was identified in a number of cases. Dark hyphae normally were confined to the outer layers of the rhytidome, but in one sample (BG-91-3), were seen in the deepest phellem layer of this tissue, close to the cortex.

Unidentified pycnidia with small spores were also common on, or slightly embedded in, the outer bark. These were likely from a variety of different fungi, since pycnidia ranged from 45 x 50 µm to 64-100 x 70-115 µm. Spores were globose or oblong and from <1 µm in size to 1 x 2-3 µm. Stalked apothecia of Phaeocalicium populneum (Brond. ex Duby) A. Schmidt (Tibell 1975), a bark saprophyte on poplar (Fig. 13B), and pseudothecia, possibly of Melanomma sp. (Fig. 14B), which occurs on dead wood of various broad-leaved trees (Dennis 1978; Breitenbach and Kränzlin 1984), were common on small branch galls.

Bacteria were not obvious in gall tissue. Of the three specific stains used for this purpose, the toluidine blue technique of Preece et al. (1979) and Sakai (1973) was the most reliable because the coloration of lignified and non-lignified cell wall layers and other features was exactly as expected. Therefore it is likely that bacteria would have been obvious if present in large numbers. Indirect evidence for bacterial presence,

though, was seen in three stem galls. In tangential longitudinal section, the pit borders of xylem vessels were frequently mottled, presumably because circular or oval holes were present in the pit borders (Fig. 14C); in contrast, normal vessel pit-borders always stained an even green. The multicolored walls of gall vessels indicated that they were chemically different from normal. Degradation of pit borders is a common form of damage caused by bacteria in wood (Eriksson et al. 1990; Greaves 1969).

Fig. 14. (A) Dematiaceous hyphomycetes common on surface of thickened bark of all galls contribute to the black color of these tumors. (B) Fruiting body and spores, possibly of Melanomma sp., common on the surface of small branch galls from Blue Ridge. (C) Mottled pit borders (arrows) on vessel walls (TLS) in three of the stem galls examined by microscopy may indicate bacterial degradation. Vessel element is short and orientation is abnormal (perforations would be at left and right sides). Scale bar = 10 μm.


A
B
C

Discussion

Summary of major anatomical features of black galls and comparison with other galls

Xylem

Black galls were found to be hyperplastic tumors consisting mostly of recognizable vascular tissue. Greatly increased frequency of cell divisions in the vascular cambium of galls resulted in more cells per growth ring than on the non-gall side of the stem and a higher proportion of fibers to vessel elements. This heightened cambial activity also led to an imbalance between the rates of cell division and differentiation of the new derivatives. This imbalance resulted in a wider cambial zone and a prolonged juvenile state of xylem derivatives (Fahn and Werker 1990). The accumulation of these frequent divisions affected the longitudinal orientation of wood cells, creating wavy grain, cell whorls, and multiseriate rays. These anatomical features are not peculiar to black gall of aspen, but also occur in many other woody galls and in response to trauma. Whorls of cells, for example, occur in reestablished cambium adjacent to frost rings in apple and mountain ash (Zalasky 1972a, 1972b, 1976), in lesions caused by the tarnished plant bug (Lygus lineolaris (Palisot) de Beauvois) in hybrid poplars (Juzwik and Hubbes 1986), in Cronartium quercuum (Berk.) Miy. ex Shirai galls on shortleaf pine (Jewell and Walker 1967), in woody tumors of white spruce (White and Millington 1954), and in Peridermium galls, likely those of C. quercuum or Endocronartium harknessii (J.P. Moore) Y. Hiratsuka, on pine (Stewart 1916). Whorled cells result because rapid cell division of fusiform initials causes an accumulation of many cell-ends, which then

become forked as a consequence of intrusive growth (Wloch 1976). These branched initials are an obstacle to normal growth of other cells, which become bent. Ray cell height in black galls was much reduced, as seen in tangential section (Table 3), perhaps because rays were split by the intrusion of fusiform cells between the radial walls of ray parenchyma (Esau 1965; Wloch 1976).

The short, small-diameter vessels in gall wood probably ensure that some water conduction can occur, since large-diameter vessels, such as those occurring in ring-porous tree species, are more readily embolized (Zimmermann 1983). Vessel elements are typically shorter in similar growths such as lignotubers than elsewhere in the same tree species (Carlquist 1988), and this is probably an essential characteristic in gall-like woody structures if water transport is to occur. Water conduction in gall wood will be considered in more detail later.

Many types of plant galls contain hypertrophied (greatly enlarged) cells. For instance, in bacterial galls of olive and oleander, abnormal cell enlargement occurs surrounding cavities filled with bacteria (Pseudomonas syringae) and cellular debris (Sinclair et al. 1987; Wilson and Magie 1964). Unusual cell enlargement was not seen in phloem or xylem of black galls.

The abnormal growth of black galls is undoubtedly related to a hormonal stimulus. Organisms that cause plant galls, such as fungi, bacteria, nematodes, and insects, release chemicals that support the development of galls (Kaiser 1981), or pathogens may stimulate host cells to secrete unusual quantities of hormones that support gall formation. For example, cytokinin activity in loblolly pine is 10 times greater in fusiform-rust-

induced gall tissue than in noninfected tissue, presumably because of a complex interaction of fungus activities and host growth (Rowan 1970). In black knot of cherry, Apiosporina morbosa produces indoleacetic acid (IAA) to stimulate hypertrophy, and another chemical stimulus, probably a cytokinin, induces cell division (Greene 1962). Several growth-regulating substances act synergistically in gall production (Braun 1969). Biochemical studies are in progress to determine the hormonal status of black gall tissue (G. Herger, personal communication).

Cambium

Although nuclei in gall cambium appeared to be larger than in normal cambium, it is not possible to speculate on their comparative ploidy levels on the basis of this observation. Nuclear size is not only affected by chromosome number but also by chromosome volume, the amount of nuclear sap, time of day, and tissue type (Esau 1965). In some tree species, nuclei are more elongated and larger just before the onset of seasonal activity (Catesson 1990). The greater frequency of divisions in gall cambium at any given time might account for the larger chromatin bodies in this tissue. Mellerowicz and Riding (1992) found that nuclear size decreased and the chromatin condensed in secondary phloem and xylem cells of Abies balsamea. In this study, a similar reduction was observed in maturing derivatives of both normal and gall cambium.

Gall initials also appeared to be more often multinucleate than did normal initials, although tangential sectioning alone may provide insufficient evidence of multinucleate initials, since sections generally contain several layers of very thin-walled, radially

flattened cells that are superimposed (Catesson 1990). Thus a radial tier of several uninucleate cells may appear as one multinucleate cell.

Phloem

Anatomical descriptions of gall phloem are much less common in the literature than are descriptions of gall xylem. This may reflect the greater difficulty in identifying the various phloem elements. Most studies of woody galls refer only to an increased percentage of parenchyma in the phloem, enlarged cells, or tissue disorganization (Peterson 1960; Jewell et al. 1962; Jackson and Parker 1958; Sinclair et al. 1987). Less cellular differentiation and smaller axial elements were characteristic of black gall phloem. Underdeveloped sieve elements and few starch plastids may indicate malnourishment of these tissues during formation, with large diversion of resources to cell division and defensive reactions by living cells and away from cell expansion and differentiation. Certain features may also reflect a water deficit in these tissues, since, as indicated below, water conduction was likely greatly reduced in gall tissue. For example, in some woody angiosperms, phloem fibers undergo secondary wall thickening at the end of the growing season (Evert 1963); the maturing of these fibers unusually close to the cambium in black galls may imply that late-season conditions, e.g. lack of water, occurred throughout the period of cambial activity. Or, secondary growth may have resulted from hormonal imbalances in these tissues. Evert and Kozlowski (1967), in experiments with bark removal from aspen, concluded that phloem and xylem differentiation in isolated tissues was less related to food availability than to hormonal influences.

Wood callus and dead cambium

Although recognizable, but abnormal, cells typical of aspen sapwood constituted most of the gall, there appeared to be a gradation from undifferentiated callus near localized areas of dead cambium to almost completely normal tissues in white islands. Carlquist (1988) referred to bands of large parenchyma cells in xylem as "pith flecks", and stated that they represent a traumatic condition in which the cambium has been injured locally, for instance by cold and drought injury. It is not unusual for such callus cells to become sclerified, as was seen in this study. However, if the areas of dead cambium were caused by a single event such as a wound, callus tissue would be produced from the exposed surfaces, eventually grow into the wounded area, and new meristematic tissue cells would form through the callus, continuous with the surrounding intact cambium (Esau 1965; Mullick 1977). In black galls, the strands of callus frequently extended through many successive growth rings, implying that the tree is unable to heal these wounds and that a continuing or repeated source of damage is present in the same part of the gall year after year. A similar situation was observed by Allen et al. (1990) in resistant lodgepole pine infected with western gall rust (Endocronartium harknessii (J.P. Moore) Y. Hiratsuka. A cone-shaped zone of pathological tissue extended through a disrupted cambium to the pith of seedlings, and this was bordered by a necrophylactic periderm. These were considered to be latent infections, in which continued presence of the pathogen in the lesion likely prevented healing. Likewise, in black galls periderm layers separated the wood callus from rudimentary differentiated xylem. In cross sections of some galls (e.g. Fig. 6D), concentric annual rings of darkened, hyperplastic tissue

surrounded the strands of wood callus, reinforcing the notion that the gall-forming stimulus originated in these areas. If so, a chemical stimulus for accelerated cell division would have to reach living cambial cells before the formation of wound periderm (see Resistance reactions, below). At a greater distance from the stimulus, the cambium was able to function normally, and white islands resulted; in these regions, divisions occurred at a more normal pace, and reactions to infection such as vessel tylosis and occlusion, and production of large quantities of phenolics by ray parenchyma cells, were absent. These responses to infection will be dealt with in more detail below.

Resistance reactions

Trees produce non-specific defense reactions to wounds and many different infectious agents. Evidence suggests that parenchyma cells react in a similar way to all kinds of injuries, including those caused by biotic agents such as viruses, bacteria, fungi, nematodes, and insects, and to abiotic agents such as drought stress and certain chemicals (Berryman 1988; Bell 1981). Many of the structural and chemical differences between normal and gall tissue, such as thickened outer bark, vessel tyloses, suberization, and elevated levels of lignin and phenolics are protective responses that are present in wounded or infected tissues in many tree species. When these reactions successfully confine an infectious agent or protect the tree from infection during wound healing, they may be of limited duration. In black galls, these responses appeared to be ongoing. A review of the significance of these reactions based on other pathological systems may aid in an understanding of their importance in black galls.

Thickened bark, necrophylactic periderms, and suberization

The outer bark of trees is extremely important to their survival because it is the first line of defense against invading organisms and environmental stresses such as desiccation. In many aspen stands, the outer bark of large trees is smooth and does not differ appreciably from that of saplings. In smooth-barked aspen, there is little change in the thickness or composition of the periderm, the constant thickness being maintained by the sloughing off of surface phellem or cork cells and addition of new cells from beneath (Kaufert 1937). In other aspen stands, mature trees have dark, rough bark from the base to the crown or only on the lower boles. Rough bark begins to form when the first-formed periderm ceases to function (Rees and Shiue 1957-58), but the cause of this condition may be variable. Kaufert (1937) maintained that deeply fissured periderm is an abnormal condition due to fungi, such as D. tumefaciens, that limit their attack to the outer cortex, or to lichens or mechanical injury from hail or whipping of surrounding brush. Bark texture also varies among aspen clones, with increase in rough bark related to sensitivity to reduced light intensity or predisposition to disease (Barnes 1966, 1968; Steneker and Wall 1970).

The normal first periderm of an aspen tree is known as exophylactic periderm and it consists of three layers. Phellem cells become water-impervious as a result of suberin accumulation in their cell walls. These cells are dead at maturity, whereas meristematic phellogen and the underlying phelloderm remain alive. Wound periderms and sequent periderms forming the rhytidome are all the same, and are called necrophylactic periderms. They are chemically different from exophylactic periderms, and their

formation has been described in detail, based on damage to firs by balsam woolly aphid (Adelges piceae (Ratz.)) (Mullick 1977). Formation of necrophylactic periderms is an active process involving metabolic and anatomical alterations. Whenever phellogen becomes non-functional, regardless of the cause, the process of phellogen restoration is initiated. Pre-existing cortical or phloem cells become dedifferentiated to assume a meristematic function and become a phellogen, a process that may be initiated by substances released from injured cells (Mullick 1977; Puritch and Jensen 1982).

Structural responses such as formation of necrophylactic periderms serve as physical and chemical barriers that are important defence reactions. These defense processes appear to be nonspecific in both angiosperms and gymnosperms (Biggs et al. 1984), with the chemical changes following a specific order. In response to wounds and infection, accumulation of phenolic compounds and lignification of cell walls precedes suberization, suggesting that a chemical signal released during wounding initiates the reactions leading to suberization (Rittinger et al. 1987; Kolattukudy 1977,

The chemistry of suberin, which appears largely responsible for the impe. ousness of periderms, is poorly understood. It appears to be a polymer consisting of an aromatic, lignin-like structure with attached aliphatic components. Waxes associated with the polymer ensure that suberized layers are an efficient barrier to water diffusion. Unlike cutin, which is an extracellular polymer, suberin is deposited within the cell wall and outside the plasma membrane. Suberin is thought to function protectively in at least three ways (Kollatukudy 1977, 1984; Biggs 1987; Esau 1965). First, phenolic constituents of suberin may be toxic to microorganisms. Secondly, it is a barrier to outward fluid

diffusion, thus preventing tissue desiccation. Biggs (1984, 1985) showed experimentally that imperviousness in woody dicots is closely related to the deposition of suberin lamellae in boundary layers formed prior to generation of new phellogen. Thirdly, suberin may be a barrier to inward diffusion of fungal enzymes. Pearce and Rutherford (1981), studying barrier or reaction zones in wounded oak, found that only suberized tissues remained intact when challenged with the decay fungus Stereum gausapatum Fr. Similarly, Akai and Fukutomi (1980) maintain that suberin is an inhibitor of tissue maceration, specifically inhibiting pectate lyases.

Although few pathogens can penetrate suberized wall layers, some can use this substance as the sole source of carbon (Kolattukudy 1984). This may be true of the fungi growing inside empty suberized cells of the rhytidome of black galls. Such fungi were not seen, however, within the adjacent lignified, phenolic-rich layers. These fungi may have been able to circumvent the layers containing toxic compounds and gain access to phellem cells through bark fissures.

Where thickened rhytidome occurred on the non-gall side of Tree 102, the layers of heavily lignified cortex and phloem that alternated with periderms were much thinner than in the case of gall tissue, perhaps because of the greater number of cells produced in gall phloem. Therefore, although the types of defensive substances (lignin, phenolics) in the rhytidomes of normal rough bark and gall bark may have been the same, the amounts of these substances may have been different.

The cause of phellogen death and the resulting formation of multiple periderms at successively deeper regions of the cortex and phloem over black gall xylem is

unknown. The surface fungi, which were always present on outer gall bark, were thought to be saprophytes, since this tissue was dead and far from deeper living cells. Only in one sample (BG-91-3) were fungal hyphae observed in the phellem cells just outside of the most recently formed phellogen layer. Although surface fungi may cause an irritation that is responsible for the thickened outer bark, their presence is not likely related to tumor formation.

In black galls, layers of cells with suberin-lined walls were present not only in rhytidome, but separating dead cells of damaged cambium from living, rapidly dividing callus, and separating wood callus from differentiated gall sapwood. This is consistent with reported descriptions of periderm formation resulting from an injury that extends to the sapwood (Mullick 1977; Biggs et al. 1984). All living tissues in the central area die, phellogen restoration occurs in a roughly cylindrical shape from the exophylactic phellem to the sapwood, and sapwood impermeability results. Any injurious agent would be totally isolated from both the bark and the sapwood. Thus if the chemical stimulus to excessive cell division arises in these wounded areas, it must be capable of diffusion through the necrophylactic periderm, or more likely, it must influence sapwood formation before the periderm is formed.

Lignin and phenolics

Many organic compounds that may be fungitoxic or fungistatic appear in the bark and sapwood of trees after wounding, injury, or microbial attack. They may accumulate in narrow reaction zones, or be a more generalized response, such as seen in black gall

sapwood. Some authors consider these compounds to be phytoalexins, since they are not preformed and are produced by dying parenchyma cells (Hart and Shrimpton 1979; Kemp and Burden 1986). The processes leading to accumulation of these substances appear to be similar to those involved in heartwood formation, although quantities of specific substances may differ (Hart and Shrimpton 1979).

The large quantity of phenolic compounds and enhanced lignification in black galls may confer decay resistance on these tissues and may be a reaction to an unknown pathogen or pathogens. Lignification and production of phenolic compounds may be linked through common precursors via the shikimic acid pathway, which is stimulated in injured or infected plants (Vance et al. 1980; Blanchette and Biggs 1982).

In healthy sapwood, lignin forms a normal part of the middle lamella and primary cell walls, with lesser amounts in secondary walls (Esau 1965; Akai and Fukutomi 1980). It protects nutrient-rich cellulose and hemicelluloses from microbial enzymes of all but the most specialized fungi. Lignin may hinder fungal growth through plant tissue by making walls more resistant to mechanical penetration and to dissolution by fungal enzymes; by restricting diffusion of enzymes and toxins from fungus to host, and of water and nutrients from host to fungus; by inactivating fungal membranes, enzymes, toxins, and elicitors with low-molecular-weight precursors; and by lignification of hyphal tips (Ride 1980).

Lignin is a phenolic polymer formed mostly by the free radical condensation of hydroxycinnamyl alcohols. Studies of the protective ability of induced lignification have been conducted mainly on herbaceous plants such as wheat and reed canarygrass, where

it was found to be an important component of leaf papillae; these cellular appositions, which are produced in response to hyphal penetration, resist digestion by fungal enzymes. In these situations filamentous fungi appear somewhat specific in their ability to induce lignification; yeasts and bacteria are poor elicitors of lignification (Bell 1981; Vance et al. 1980; Pearce and Ride 1980). It is not known whether this information may be applied to woody plants. For example, the total phenolic content of willow infected by the bacterium Erwinia salicis was double that of healthy wood (Wong and Preece 1978) and phenolic compounds were increased in bacterial disease of ash (Janse 1982). Increased lignification was not reported in either case.

The colored phenolic substances observed in black gall sapwood and bark most likely consist of ortho-quinones and tannins. The enzymes polyphenoloxidase (PPO) and peroxidase (PO) oxidize the colorless dihydroxyphenols to give the colored ortho-quinones. Polymerization of certain dihydroxyphenols also produces tannins. Tannins and ortho-quinones are toxic to most microorganisms and viruses (Bell 1981). Fungal hyphae may be absent from the heavily lignified, phenolic-containing cells of gall rhytidome partly because of the fungitoxic nature of these substances.

Tyloses and vascular occlusion

Coincident with the above chemical changes were structural modifications of gall sapwood, especially vessel elements. Dessureault and Tattar (1975) provided direct evidence that passive flow of liquids does not occur from clear to discolored maple wood. Likewise, the occlusion of vessel elements by tyloses and other unidentified fibrous

material suggested that it is unlikely that significant water conduction occurred through black gall xylem. The sap exudate present on the cut surface of only the most recent growth ring of some black galls may confirm this. Microscopically this area of young sapwood was free of tyloses and other occlusions unless collected late in the growing season. Water columns under tension are very vulnerable to cavitation, which can be induced by any kind of disturbance, mechanical stress, or microbial wall degradation (Zimmermann 1983). Tyloses form when vessels become gas-filled, and they are more common in large earlywood vessels, which are more readily embolized (Zimmermann 1983). Portions of ray parenchyma cells, including cytoplasmic contents, distend into adjacent vessel elements in response to loss of vessel water, eventually totally occluding the vessel. Together with gums, which also originate in ray cells, tyloses provide a very effective mechanism to seal off living tissue against injuries and dead tissues (Zimmermann 1983). In some tree species such as Robinia pseudoacacia L., tyloses and gums normally occur in the early wood in the fall of each year. This is not true of trembling aspen, in which healthy sapwood can remain functional for many years. In black galls, however, it is possible that the cell deformities caused by the hyperplastic condition of these tissues cause embolism to occur readily. Air intake at the points of cambial damage could also be a contributing factor to cavitation in adjacent vessels. The resulting water stress may directly cause the death of adjacent ray cells and the corresponding conversion of sugars to phenolic compounds (gums) in the same way that moisture stress has been postulated to cause heartwood formation (Hillis 1977).

The nature of the dense material occluding some vessels in black gall wood is unknown. In some sapwood infections of angiosperms, gummy deposits in vessels contain polyphenols (Pearce 1987), but the substance in black gall wood did not stain with any of the histochemical reagents used. Vascular gels that coat the walls and fill lumina of infected vessels have been found in numerous plant species infected with fungi, and they prevent movement of hyphae and spores. They have been described as a net-like matrix that fills vessel lumina and originates from perforation plates, end-walls, and pits (VanderMolen et al. 1977). Alternatively, gels may be new carbohydrates and other materials synthesized and secreted into the vessels (Bell 1981). Regardless of origin, occlusions in gall vessels would further prevent water flow through this tissue, and may also obscure presence of bacteria or fungi in these cells.

Calcium oxalate crystals

Calcium oxalate is the most commonly occurring insoluble calcium salt found in the plant kingdom. The shape of calcium oxalate crystals is variable depending on the cell type in which they form, location in the plant body, and plant species (Horner and Zindler-Frank 1982). This was confirmed for normal aspen, in which crystals were confined to phloem tissue--druse rosettes scattered throughout phloem parenchyma, and prismatic crystals confined to special vertical parenchyma surrounding groups of phloem fibers and sclereids. Various explanations (largely unproved) have been proposed for their formation: (1) a mechanism for removal of oxalate, a possibly toxic end-product of metabolism; (2) a mechanism for removal of excess calcium from the system; (3) a

form of osmoregulation, since excess oxalic acid may be formed to neutralize hydroxyl ions generated by nitrate assimilation; (4) a storage form for calcium or oxalate; (5) protection against foraging animals; or (6) mechanical support (Franceschi and Horner 1980).

Although these crystals are not generally pathological (Franceschi and Horner 1980), their presence in darkened tissues of gall sapwood and small branch galls but not in normal aspen sapwood may be noteworthy. Oxalic acid is secreted by certain fungal plant pathogens such as Sclerotium rolfsii Sacc. in sugarbeets (Punja and Jenkins 1984) and Leucostoma cincta (Pers.:Fr.) Hohn. in peach bark (Traquair 1987), and is thought to aid infection by lowering the pH of plant tissues to the optimum required by fungal polygalacturonases or by chelating calcium ions and weakening cell walls. Since calcium oxalate crystals in gall tissue invariably occurred as intracellular inclusions, it is unlikely that they originated from a pathogen, but rather from disrupted metabolism of host cells. In some infections calcium oxalate crystal formation is associated with dissolution of middle lamellae, which consist mostly of calcium pectate (Punja and Jenkins 1984). In black galls, degradation of lamellae was not obvious by light microscopy.

Crystals are often seen in callus tissue of wounds, and calcium ions are known to affect various properties of biological membranes; it is also possible that rapidly dividing meristematic tissues, typical of black galls, would not be able to cope as effectively with large influxes of ions as would mature tissues (Franceschi and Horner 1980). Beckman (1980) noted that plasticizing of cell walls is essential for the formation of tyloses, i.e. calcium must be removed to permit mature walls of parenchyma cells to distend through

pit apertures. A combination of these factors may explain the calcium oxalate crystals seen in sapwood of black galls.

Nature of causal agent

Because of the lower incidence of advanced decay in gall trees, it would obviously be of interest to determine the causal agent(s) of black galls on aspen. The association of most galls with a branch stub supports the involvement of an infectious agent in their formation, since there is some evidence that these are common entry points for pathogenic organisms in aspen (Hiratsuka et al. 1990). These galls are persistent, and continue to grow and increase in size by yearly increments along with the tree. The presence of white islands of fairly normal tissue within actively growing galls suggests that the cambium can occasionally recover from the tumorous condition, and that a persistent agent is required for continuing abnormal growth. The results of this study provide a framework for further investigations of the etiology of black galls on aspen, and a discussion of the possibilities follows.

Fungi and bacteria

In most galls caused by fungi, hyphae are easily seen growing between or within cells. This is true of Endocronartium harknessii, causing western gall rust of hard pines, in which the parasite is unable to influence host anatomy beyond the invaded tissue (Jewell et al. 1962; Jackson and Parker 1958; Allen et al. 1990); and of tumor disease of oak and hickory, supposedly caused by Phomopsis sp. (Brown 1938; Sinclair et al. 1987).

It is likely that a fungus causing extensive and continued hyperplastic growth, as is present in black galls, would be obvious in a light-microscope study. Also, given the above evidence on the barriers to diffusion presented by necrophylactic periderms and associated lignified tissues within bark and sapwood, it is unlikely that tumor-stimulating chemicals diffuse to the cambium from the fungi on the outer bark of these galls. Diplodia tumefaciens, a known gall-causing fungus, was collected from one gall bark fissure near living tissue and may be an exception to this. However, the rare occurrence of this organism in the material studied suggests that its presence may be incidental. Numerous species of fungi and bacteria have been isolated from black gall tissue, including several that are unique to these tumors: Hyphozyma lignicola (Hutchison et al. 1993), Phoma etheridgei (L. Hutchison, personal communication), and Capnohughsia nigra (L. Hutchison, personal communication). Inoculations of candidate organisms onto aspen trees will be necessary to determine whether they play a role in tumor formation.

In most bacterial cankers and galls on trees, such as galls of olive and oleander, cavities filled with bacteria and cellular debris are present in the diseased tissues (Janse 1982; Sinclair et al. 1987; Wilson and Magie 1964). No such cavities were seen in black galls. Although the mottled pit borders in gall vessels are indicative of bacterial presence, this erosion may be secondary, and not a primary cause.

Is it possible that black galls are caused by Agrobacterium tumefaciens? Unlike many other bacterial plant diseases, in which tissues are killed and disintegrate, the crown-gall bacterium (Agrobacterium tumefaciens) stimulates parenchyma cells to abnormal growth. Bacteria must enter the host through a wound, where they transform

host cells by releasing plasmid DNA that codes for tumor production. Transformed tumor cells produce abnormal concentrations of auxins and cytokinins and multiply autonomously. Increase in cell size and rapid divisions result in gall formation (Braun 1982; Dowson 1957). Most poplar species are susceptible to the crown gall bacterium (Riffle and Peterson 1986), and aerial galls are common on these species (Sinclair et al. 1987). Descriptions of the anatomy of these galls, however, are often imprecise or contradictory. According to Dowson (1957), galls are generally soft on herbaceous hosts, but on woody stems they are always hard, and consist of a disorganized mass of parenchyma and vascular elements, with the latter predominating. However, Ostry et al. (1989) and Sinclair et al. (1987) state that these deeply fissured growths can later become spongy on poplar, or are initially spongy, becoming hard later as disorganized clusters of xylem elements differentiate in them. Bacteria may live and multiply on the outside of the gall, but are not present deep within it, and may be especially difficult to isolate from woody galls (Dowson 1957). Bacterial slime may appear in fissures, however, when weather is humid (Ostry et al. 1989). Except for the presence of spongy tissue, the anatomy of black galls is consistent with this description.

The occurrence of white islands of seemingly normal tissue within black galls is also possible in crown galls. Several investigations, particularly with tissue cultures, have shown the phenotypic reversal of crown gall tumorigenesis. Although the mechanism of reversal is not completely understood, it may involve the loss or reduction of bacterial plasmid DNA by the host cells (Gordon 1982).

The ability of bacterial diseases to become systemic and establish secondary tumors in other plant parts is well-documented for oleander tumors induced by P. savastanoi (Wilson and Magie 1964; Wilson 1965) and for crown gall tumors caused by Agrobacterium (Turgeon 1982). A systemic bacterial disease could explain the extensive galling of both stem and branches.

Several genera of bacteria have been isolated from black galls, with the most common being Pseudomonas sp. and Bacillus sp. An isolate of Agrobacterium sp. was obtained only from one gall-like growth at the base of a tree at Edson (G. Herger, personal communication). This gall was the only one found at soil level in any of the plots examined, and it had a more irregular shape than typical aerial black galls.

Insects and mites

The finding of large stem galls with active poplar budgall mites prompted a search for reports of this phenomenon in the literature. Although Campbell et al. (1969) stated that A. parapopuli rarely infects aspen stems, they reported finding large stem galls at least 12 years old caused by these mites; it is not clear whether mites were actually recovered from these galls. Ives and Wong (1988) claimed that most mite galls contain mites for 1-4 years, but others may be occupied for 10 to 15 years, implying that fairly large galls are possible. In this study, galls from Hinton suspected to be caused by mites were 30-40 years old.

Gall-causing mites are almost exclusively from the family Eriophyidae, of which A. parapopuli is a member, and they are usually host-specific (Jeppson et al. 1975; Baker

and Wharton 1952). Since salivary secretions of the mite provide the stimulus for hyperplastic growth (Campbell et al. 1969), it is assumed that continuous abnormal tissue growth depends on their presence (Kaiser 1981). However, some studies cast doubt on this assumption. Jeppson et al. (1975) stated that eriophyid mites produce particular regulators that cause formation of galls of specific benefit to that mite. After the induced change has altered the behavior of the affected cell or cells, the mite does not have to remain on the site to insure continuation of gall growth. Early-season galls start development before they are inhabited by mites. Later, when the brood has increased, the mites move into the prepared galls. Likewise, a few mites appear to inject a systemic and persistent toxin or growth regulator into their host. This causes a disruption or increase in localized growth at some distance from the feeding area. For example, Eriophyes tulipae Keifer, besides transmitting viruses and other diseases, may inject a systemic toxin into corn plants and some grasses. Brevipalpus mites on citrus and other plants introduce a persistent systemic toxin into the plant during the feeding process. Tandon et al. (1987) reported that galls incited by the mite Eriophyes cernuus on Zizyphus jujuba Mill. have transplantable and tumefacient properties. Unregulated synthesis of auxin protectors (o-dihydroxyphenols) may be responsible for hyperauxinity and growth autonomy of the gall tissue. It is possible that the type of galls found in the study plots in Central Alberta were also mite-caused, but careful dissection of outer bark did not reveal similar mites or succulent deformed tissue, a necessary prerequisite to growth and reproduction of eriophyid mites (Westphal 1992). The different gross morphology, both internal and external, of the four black galls used for the anatomical study, and the occurrence, on the

same trees, of small branch galls with no resemblance to poplar budgalls, strongly support the notion that these black galls have a different origin.

The distinguishing feature of insect galls is their determinate growth (Braun 1969; Harper 1959). Results of tissue culture studies suggest that cells of insect galls are transformed from their normal growth pattern only in the presence of the insect, or as long as its secretions are present (Kaiser 1981). They are of constant form and size and possess their own polarity and symmetry (Braun 1969). In poplar, the most common insect galls are on leaves or petioles, such as those caused by aphids of the genus Pemphigus (Harper 1959). Although large insect galleries were found in one black gall tree, these did not seem to be directly related to the large stem galls found on this tree. There was ample evidence of mites and insects in the outer bark of galls. The sap exudates appeared to be related to physical damage in the cracks and fissures, and empty insect cases were often seen. Obviously, black galls provide a home for many creatures. The possibility of mites or insects as gall initiators or as vectors of a gall-forming agent cannot be eliminated.

Viruses and mycoplasma-like organisms

Very little work has been done on tumorigenesis by plant viruses, and most studies have involved wound tumor disease, particularly in sweet clover (Kaiser 1981). The most commonly reported virus disease symptoms in trees are leaf mottling, spotting, vein-banding, and other deformations; twig stunting and degeneration; stem necrosis, rough bark, gummosis, or pitting. Virus concentration may be low in woody hosts, and

detection may be further hindered by the presence of large amounts of tannins and phenolic substances (Nienhaus and Castello 1989). Eriophyid mites are known vectors of many plant viruses (Jeppson et al. 1975), and viruses are widespread in forest ecosystems, including soil (Nienhaus and Castello 1989). Mycoplasma-like organisms (MLO's) are responsible for tree diseases with such symptoms as yellowing, stunting, witches' broom, phyllody, and abnormal fruits and seeds. In elm phloem necrosis disease, caused by a MLO transmitted by a leaf hopper, sieve tube necrosis is accompanied by hyperplasia and hypertrophy of adjacent parenchyma (Schneider 1973; Nienhaus 1985). However, in the absence of extensive information on woody tumors caused by these agents, there remains a strong possibility that black galls are the combined result of insect or mite activity and a viral or mycoplasmal pathogen.

Clues from branch galls

The presence of numerous small branch galls on trees with large stem galls and the tissue similarities between galls of all sizes provides circumstantial evidence that they have a common origin. On the knob-like branch galls the brown dead tissue extending from outer bark to the xylem of these galls was similar to the wood callus of large stem galls. Likewise, the presence of knob-like branch galls in association with stem galls having large bark excrescences, but not with the type bearing mite-containing bud proliferations, supports the theory that there are at least two distinct causes of large stem galls on aspen.

The centers of branches bearing small galls were degraded, and dark fungal hyphae and spores were common. However, these may not be related to gall formation, since similar browning and decay was often seen in branches without galls. The close association between leaf traces and these fungi seen in sections parallel to the branch axis, along with their presence at leaf scars, may indicate that these fungi gain entrance to the twig center through the leaf scar. Wilson and Magie (1964) showed that on olive, the scars formed when leaves fall are common infection sites, remaining susceptible to infection by bacteria for 1 week after leaves fall. Such infection may be the ultimate cause of twig pruning so common in aspen. Isolation and identification of the organisms present in many of these small branch galls, comparison with those in twigs of non-gall trees, and finally, inoculation experiments, will be important in determining the etiology of black galls on aspen.

CHAPTER IV. GENERAL DISCUSSION AND CONCLUSIONS

Nature of tumorigenesis in black galls

The anatomical study of black galls on aspen showed that these structures consist mostly of the same recognizable cell types as healthy aspen, but that the tumor results because of more frequent cell division in the cambium. In addition to hyperplasia, black galls are sites of localized structural and chemical modifications that are normally associated with responses to wounding or infection. These include thickened bark consisting of successive necrophylactic periderms, enhanced lignification, development of suberized cork layers adjacent to damaged areas, production of large quantities of phenolic compounds, and formation of tyloses and granular obstructions in vessels. The reactions in sapwood occur within a year of new cell production, and seem to correspond in time and place with the death of xylem ray cells. The source of yearly damage to the cambium and its seeming inability to recover, sometimes for several years, is a puzzle. Dead and callused regions are surrounded by the most intense resistance reactions noted above. These areas, along with white islands, where both resistance reactions and abnormal cell proliferation are absent or greatly reduced, suggest that a persistent agent is needed for continued tumor growth in galls having these characteristics. White islands and disrupted cambium, however, were not present in all 14 galls for which gross anatomy was studied. It is possible that some black galls are self-limiting (tissues can recover normal growth), whereas others are non-self-limiting (a permanent genetic change has occurred in the meristematic cells of the gall). There is a need for further studies of the etiology of these tumors.

Although the causal agent of black galls was not established, the anatomical study suggests several possibilities. It is unlikely that the cause is a fungus or bacterium, since galls caused by these agents generally contain these organisms in large numbers. The discovery of large stem galls at Dawson Creek caused by the poplar budgall mite explained some of the morphological diversity seen in aspen stem tumors deposited in the herbarium of the Northern Forestry Centre. This finding, along with the physical damage seen in the cambium of both stem and branch galls, strongly implicate mites or insects in black gall etiology. The following features of the galls in the study plots, however, make it unlikely that the mite A. parapopuli is the cause of these: gall origin from a discrete point (e.g. branch stub); absence of fresh buds on the gall surface or of old buds inside the old sapwood; more obvious growth rings than in budgalls; and the presence of small branch galls on two trees from Blue Ridge that are very different in morphology from galls caused by this mite. It is also possible that mites or insects initiated tumorigenesis early in the life of a tree by introducing a virus, mycoplasma-like organism, or Agrobacterium tumefaciens, which has resulted in continued gall growth. None of these microorganisms would be visible in a light microscope study.

Apart from the small branch galls, the material used for this study was from old tumors. Diseases caused by different causal agents that induce different primary symptoms may become similar as the diseases progress (Schneider 1973). Thus, although the four stem galls used for the anatomical study shared similar structure and histochemistry, it is possible that they were caused by different agents.

Black gall and aspen decay

While the anatomical study provided some interesting etiological possibilities, the field study confirmed Hiratsuka and Loman's (1984) hypothesis that aspen trees with black galls are less likely to have advanced decay by P. tremulae than are surrounding trees. Systemic resistance related to the presence of black galls or their causal agent is a plausible explanation for this phenomenon. Logistic regression also confirmed previous studies that the likelihood of a given tree having a conk was related to the site and tree diameter (Hiratsuka and Loman 1984; Wall 1971; Maier and Darrah 1989).

Comparison of individual plots showed a difference in the degree of the protective effect related to presence of black galls. Further field studies are needed to determine whether all trees in a given plot were from the same clone, and whether variability in biochemical response by clones to wounding or pathogenic organisms accounts for the differences among plots in degree of the protective effect associated with black galls.

The increasing demand for aspen wood and pulp products has created a need for greater understanding of the decay and stain that hinder the efficient utilization of this resource. Knowledge of the factors that control susceptibility and resistance of aspen to disease may eventually lead to an ability to enhance its natural defense systems against pathogen attack. This may include biological protection strategies to prevent colonization or attack by pathogens (Zabel and Morrell 1992). The recent isolation from aspen of fungi antagonistic to blue-staining and decay fungi, and identification of their fungitoxic compounds (Chakravarty and Hiratsuka 1992, 1993; Hiratsuka et al. 1990, 1993; Ayer and Miao 1993; Trifonov et al. 1992) provide concrete material for testing of strategies to

protect aspen from these pathogens. In view of the strong resistance responses, both localized and systemic, shown by trees with black stem galls, these tumors may be sources of biological protective agents as well as genetic material for genetic engineering and traditional breeding of aspen clones less susceptible to serious decay.

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APPENDIX: GLOSSARY OF ANATOMICAL TERMS USED TO DESCRIBE WOODY TISSUE

Bark terminology is largely based on the recommendations of Trockenbrodt (1990) and Martin and Crist (1970); xylem terminology on Carlquist (1988) and Esau (1965, 1977). Other sources are indicated where necessary.

Bark: All tissue outside the vascular cambium. Inner bark includes the tissues between the cambium and the last-formed periderm or protective layer; outer bark (rhytidome) includes the layer of dead tissue of a dry, corky nature outside the last-formed periderm.

Burl: Any anomalous or unusual woody structure with a swirled grain (James 1984); also used to describe woody tissue around stem wounds of trees and shrubs (Zalasky 1975a, 1975b).

Cambium/cambial zone (vascular): The lateral meristem that forms the secondary vascular tissues, phloem and xylem, in stem and root. In storied cambium, short fusiform cells are in horizontal tiers; in nonstoried cambium, fusiform cells are long and overlapping.

Chromatin body: The part of a nucleus that becomes aggregated into chromosomes during nuclear division.

Companion cells: Specialised cells that are closely related to sieve tube members in a physiological sense; they have a role in the transport mechanism.

Cork: See phellem.

Cortex: Bark tissue of primary origin that belongs neither to the epidermis, the periderm, or the phloem. In this study, it refers to the parenchymatous region between the last-formed periderm and the phloem.

Diffuse-porous wood: Secondary xylem in which the vessels are distributed fairly uniformly throughout a growth ring or change in size gradually from early to late wood.

Druse crystal: Spherical rosette-like aggregate of individual calcium oxalate crystals (Franceschi and Horner 1980).

Fiber: A long, slender cell with apical growth and thickened walls; directly differentiated from meristem derivatives.

Fusiform initial: In the vascular cambium, an elongated cell that gives rise to the axial system in the secondary vascular tissues, e.g. fibers, vessels, sieve tube elements.

Gall: Localized overgrowth in which host cells are stimulated to excessive growth by a variety of disease-producing agents (Braun 1969).

Hyperplasia: Excessive multiplication of cells.

Hypertrophy: Abnormal cell enlargement.

Leaf papilla: A localized cell wall thickening in response to penetration of a cell by fungal hyphae. Papillae contain substances such as lignin, which are thought to function in host resistance (Ride 1980).

Libriform fiber: A xylem fiber with simple pits.

Lignotuber: A woody swelling found at the stem base of certain tree species; it contains dormant buds, and carbohydrates and nutrients necessary for development of these buds (James 1984).

Meristematic cell: A cell that produces new cells by division.

Middle lamella: The layer of intercellular material, composed mainly of pectic substances, that cements together the primary walls of adjacent cells.

Parenchyma cell: A cell that retains a living protoplast at the end of its ontogeny; it is typically brick-shaped or isodiametric and has simple pits (Trockenbrodt 1990). Boundary apotracheal parenchyma is axial xylem parenchyma not associated with vessels and occurring only as a band at the end or beginning of a growth layer.

Perforation (of vessel element): The simple or compound opening where vessel elements join to form a vessel.

Periderm: A protective tissue of secondary origin that replaces the epidermis in stems and roots that have continual secondary growth (Biggs et al. 1984). Exophylactic periderms protect living tissue from external environmental factors. Necrophylactic periderms protect living tissue from being affected by the death of adjacent cells.

Phellem: Suberized protective tissue that develops outward from the phellogen (Biggs et al. 1984). Also known as cork.

Phelloderm: A living parenchyma formed inwardly by the phellogen (Biggs et al. 1984).

Phellogen: The meristematic layer of bark cells responsible for the development of the periderm.

Phloem: The principal assimilate-conducting tissue, usually separated from the xylem by the cambium.

Pit: A recess or cavity in the cell wall where the primary wall is not covered by a secondary wall. In a bordered pit the secondary wall overarches the pit membrane; a simple pit has a constant width.

Pith fleck: A band of parenchyma cells, irregular in orientation, found in various woods. It represents a traumatic condition in which the cambium has been injured locally. The callus cells may become sclerosed. See Wood callus.

Prismatic crystal: A crystal resembling a prism (i.e. with faces parallel to one axis) (Franceschi and Horner 1980).

Ray: A sheet of parenchyma extending radially inward from phloem to xylem. Homocellular rays are composed of a single cell type; heterocellular rays, of two or more cell types. Multiseriate rays are more than one cell wide, as seen in tangential longitudinal section. Uniseriate rays are only one cell wide, as seen in tangential longitudinal section.

Reaction zone: A barrier zone characterized by polyphenolic materials and laid down in pre-existing, anatomically normal sapwood in response to wounding or infection (Pearce 1990).

Rhytidome: The outer bark tissue, separated from the living inner bark or phloem by the last formed periderm. It protects the underlying tissue from desiccation and other organisms.

Ring-porous wood: Secondary xylem in which the vessels of the early wood are distinctly larger than those of the latewood and form a well-defined ring in cross-section of the wood.

Sclereid: Cell with thickened and usually polylamellate secondary walls; it differentiates from another mature cell by secondary growth.

Sclerenchyma: Any tissue consisting of cells with heavily lignified, polylamellate secondary walls.

Sieve area: A distinct grouping of pores on a phloem sieve tube member. See Sieve plate.

Sieve plate: A specialized wall area, especially of the end wall, of a phloem sieve tube element showing one or several sieve areas or groupings of sieve pores. A simple sieve plate consists of only one sieve area; a compound sieve plate consists of several sieve areas.

Sieve pores: Pores that interconnect sieve elements or connect sieve elements to adjacent cells; they may be distinctly grouped into sieve areas.

Sieve tube: An assimilate-conducting tube in the phloem, consisting of interconnected sieve tube members.

Sieve tube member: In an angiosperm, a sieve element possessing sieve plates, arranged end to end to form a sieve tube.

Slime plug: An accumulation of phloem protein (p-protein) on a sieve area, usually with extensions into the sieve pores. Occurs in response to injury.

Suberin: A polymer with a lignin-like structure and associated waxes; it is deposited within the cell wall and is responsible for imperviousness of phellem cells.

Tylosis: A distension of the wall and cytoplasm of a ray parenchyma cell through the pit and into the lumen of a xylem vessel member; the cause is loss of water pressure within the vessel.

Vascular gel: A net-like matrix of carbohydrates or other materials that fills vessel lumina in response to infection (VanderMolen et al. 1977).

Vessel: A tube-like series of vessel elements specialized for water conduction.

Vessel element: One of the cellular components of a vessel.

Wood callus: Thin-walled cells developing in wood as a result of injury.

Xylem: The principal water-conduction tissue in vascular plants, characterized by presence of tracheids or vessels.